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Regulation of inducible immune gene expression in T cells compared to macrophages

by

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Declaration

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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i. Publication arising from the period of my candidature

- **LD Apps** and AF Holloway, *The Role of Chromatin in Inducible Gene Regulation in the Immune System,* Histone Class, Structure and Function, 2011 Nova Science Publishers Inc.

ii. Data arising from this thesis has been presented at the following scientific meetings

- **Apps L.D**. Sprod O.R. Poke F.S. and Holloway A.F. Mechanisms Underlying the Lineage- Dependent Switch From a Primary to Secondary Response Gene. *32nd Annual Lorne Genome Conference*. Lorne, Victoria. Poster 272. (2011).

- **Apps L.D**. Poke F.S. Hardy K. and Holloway A.F. Transcription Factor c-Rel Interacts With the Chromatin Architecture of Immune Genes to Determine Gene Expression Patterns. *33rd Annual Lorne Genome Conference*. Lorne, Victoria. Poster 107. (2012).

- **Apps L.D**. Poke F.S. Hardy K. and Holloway A.F. Transcription Factor c-Rel Interacts With Chromatin Architecture to Mediate Both Activation and Repression of Immune Genes. *Epigenetics 4th Australian Scientific Conference.* Adelaide, South Australia. Poster 101. (2012).

- **Apps L.D**. Poke F.S. Hardy K. and Holloway A.F. Transcription Factor c-Rel Interacts With Chromatin Architecture to Mediate Both Activation and Repression of Immune Genes. *EMBO Conference Series Chromatin and Epigenetics.* Heidelberg, Germany. Poster 58 (2013).

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

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Abbreviations

3’	three prime
5’	five prime
Ac	acetylation
AP1	activating protein 1
APC	antigen presenting cell
ATP	adenosine triphosphate
BAF	Brg1/Brm associated factor
bp	base pairs
BRG1	brahma related gene 1
BRM	brahma
BSA	bovine serum albumin
Ca^{2+}	calcium
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CHART PCR	chromatin accessibility by real-time PCR
CHD1	chromodomain helicase DNA binding protein
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CMPP	common lymphoid primed multipotent progenitor
CpG	5’ CG 3’
CO_2	carbon dioxide
CRC	chromatin remodelling complex
DAG	diacylglycerol
DH	DNase1 hypersensitive
DMSO	dimethylsulfoxide
DMEM	Dulbecco’s modified Eagle medium

DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNMT	DNA methyltransferase
dNTPs	deoxyribonucleotides
DTT	dithiothreotol
E. Coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,'N'-tetracetic acid
FCS	foetal calf serum
Foxp3	forkhead box P3
GAPDH	glyceraldehyde phosphate dehydrogenase
GM-CSF	granulocyte macrophage colony stimulating factor
GMP	granulocyte macrophage progenitors
H	histone
HAT	histone acetyl transferase
HCl	hydrochloric acid
HDAC	histone deacetylase
HMT	histone methyltransferase
HSC	haematopoietic stem cell
I	ionophore
IFN	interferon
IKK	inhibitory kappa B kinase
IL	interleukin
INO88	inositol requiring
IP	immunoprecipitate
IP3	inositol 1, 4, 5 triphosphate
IRAK	interleukin 1 receptor associated kinase
ISWI	imitation switch
K	lysine
kb	kilo base
KCl	potassium chloride

kDa	kilodalton
LiCl	lithium chloride
LPS	lipopolysaccharide
M	molar
MAPK	mitogen activated protein kinase
M-CSF	macrophage colony stimulating factor
Me	methylation
MEF	murine embryonic fibroblast
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
Mi-2/NuRD	nucleosome remodelling deacetylase
MNase	micrococcal nuclease
mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation primary response gene (88)
NaCl	sodium chloride
NaOH	sodium hydroxide
NEMO	nuclear factor kappa B essential modulator
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa B
NIK	nuclear factor kappa B inducing kinase
NS	non-stimulated
NTC	no template control
NuRD	nucleosome remodelling deacetylase
O.D	optical density
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCRC2	polycomb repressor complex 2
PI	PMA and ionophore
PI3K	phosphatidyl inositol-3 kinase
PIP_2	phosphatidyl inositol (4, 5) biphosphate

PKC	protein kinase C
PMA	phorbyl myristate acetate
PolII	RNA polymerase II
PTX	pentoxifylline
RHD	rel homology domain
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell park memorial institute
RT-qPCR	reverse transcription qPCR
SDS	sodium dodecylsulphide
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNF	sucrose nonfermentable
SP1	specificity protein 1
SWI	switch
TAB	tumour growth factor beta activated kinase
TCR	T cell receptor
TE	Tris/EDTA
TF	transcription factor
Th	T helper
TI	total input
TLR	toll like receptor
TNF	tumour necrosis factor
TNFSF9	tumour necrosis factor (ligand) superfamily, member 9
TNT	tris/sodium/tween solution
TRAF	tumour necrosis factor receptor associated factor
Treg	regulatory T cell
Tris	Tris(hydroxymethyl)aminomethane
TSS	transcription start site
U	units
WT	wild type

Abstract

The initiation, duration and resolution of an appropriate immune response is vital for defending the host against invading pathogens, toxins and cancerous cells. Such immune responses are reliant on the cooperative action of multiple cell types and the co-ordinated regulation of gene expression programs, which direct immune function. Inducible gene expression upon immune activation is determined by the complex interaction between transcription factors, the chromatin structure and chromatin modifying/remodelling factors at the regulatory regions of immune genes. The NF-κB transcription factor c-Rel is a key regulator of gene expression programs within the immune system. c-Rel regulates expression of cytokines and transcription factors during immune cell activation and is essential for the correct function of immune cells from divergent cell lineages. These differentiated cells are highly specialised and have a diverse range of functions requiring precise regulation of their gene expression profiles for targeted responses. This study explores the role of c-Rel in divergent lymphocyte and macrophage lineages.

c-Rel has previously been shown to instigate chromatin remodelling events required for activation of the *GM-CSF* gene in T cells. Activation involves the depletion of hyper-acetylated histones from the promoter region of the gene, mediated by the chromatin remodelling protein BRG1. This study shows that activation of the *GM-CSF* gene is also c-Rel dependent in macrophages, however the gene promoter is not marked by acetylated chromatin and no loss of H3 is detected in association with activation. These findings suggest that whilst the *GM-CSF* gene is expressed in both T cells and macrophages, activation is regulated by fundamentally different mechanisms in the two cell types. The hypothesis underlying this study is that during the process of differentiation lineage specific chromatin structure is established at inducible gene promoters and that this chromatin structure largely dictates the cooperative regulation

of gene expression through interaction with transcription factors, structural proteins and chromatin remodelling enzymes.

Using genome-wide data sets, a cohort of immune genes which are regulated by c-Rel were identified. These genes were further categorised into BRG1 independent and dependent genes. The activation requirements of these genes were examined in both T cells and macrophages to determine whether the switch in regulatory control identified for the *GM-CSF* gene is a common feature of genes that are co-expressed by these cell types, and to characterise the mechanisms underlying this fundamental difference in regulation. Data presented in this thesis demonstrates that several genes undergo switches in the requirements for gene expression between the two cell types. The analysis of chromatin structure indicates that these differences in activation kinetics are reflective of the basal chromatin accessibility established at the genes promoter regions. Furthermore, the extent of chromatin remodelling across promoter regions correlates with the magnitude of the gene transcription response. Additionally, gene expression analysis of macrophage and T cells derived from c-Rel$^{-/-}$ mice suggest that c-Rel can act as a represser as well as an activator of gene transcription.

1 Regulation of inducible gene expression in the immune system

1.1 Introduction to immune gene regulation

The initiation, duration and resolution of an appropriate immune response is vital for defending the host against invading pathogens, toxins and cancerous cells. Such immune responses are reliant on the cooperative action of multiple cell types and the coordinated regulation of gene expression programs, which direct immune function. Expression programs are driven by networks of transcription factors which bind to their cognate binding sites in the regulatory regions of genes encoding transcription factors, cytokines and other proteins required for an immune response (Wilson, Rowell & Sekimata 2009). Control of gene transcription is therefore essential for proper immune cell function, but rather than being reliant on transcription factor binding alone, an intricate and multi-layered system of transcriptional regulation is in place in eukaryotes (Lim et al. 2013; Venters & Pugh 2009).

Cytokines are small signalling proteins secreted by cells of both the innate and adaptive immune system which modulate the immune response and enable signalling between cell types (Cohen, Bigazzi & Yoshida 1974; Ghosh, May & Kopp 1998). Cytokine proteins are not usually constitutively expressed, nor stored as pre-formed molecules within the cell. Instead, in response to appropriate signals cytokine production is reliant on the transcription and translation of new cytokine proteins before cell secretion can take place (Abbas, Lichtman & Pillai 2007). During an immune response cytokine gene

expression is switched on rapidly before returning to low basal levels during immune resolution (Holloway, Rao & Shannon 2002).

Immune cells, including T cells and macrophages, produce a broad array of cytokines during an immune response. The type of cytokines produced is determined not only by the cell type, but also the stimulus and cellular environment. As a multitude of cytokine genes may be expressed at one time they often affect the synthesis and function of other cytokine genes, as well as the activity and proliferation of other immune cells. Partial redundancy exists in which some cytokines elicit similar effects. Cytokines may also act synergistically or antagonistically to one another and therefore the initiation, duration and resolution of the immune response will be reliant on the precise balance of pro-inflammatory and anti-inflammatory cytokines being expressed at the appropriate time (Abbas, Lichtman & Pillai 2007). It is now becoming increasingly appreciated that the control of cytokine gene expression is essentially modulated by the interaction of transcription factors with the chromatin platform established at the gene regulatory regions (Aung et al. 2006; Brettingham-Moore et al. 2008; Lim et al. 2013; Natoli 2009).

The activation kinetics of individual inducible genes within the immune system is important for a co-operative immune response. Inducible immune gene expression is recognised as occurring in two waves depending on the activation requirements of the individual genes (Hargreaves, Horng & Medzhitov 2009; Herschman 1991). The "first responders" to activation signals are also referred to as immediate-early genes or primary responders as an increase in gene transcription may be observed within minutes of stimulation and does not require the synthesis of new proteins to occur (Fowler, Sen & Roy 2011; Herschman 1991; Ramirez-Carrozzi et al. 2009). Late responders to activation signals or secondary response genes display delayed activation kinetics and are reliant on de novo protein synthesis before increased gene transcription can occur (Ramirez-Carrozzi et al. 2009; Yamamoto & Alberts 1976). Many transcription factors are encoded by primary response genes which are required for subsequent activation of secondary response genes (Fowler, Sen & Roy 2011). A further group of genes are

classified as delayed primary response genes (Tullai et al. 2007). Like immediate-early primary response genes, delayed primary response genes are expressed independent of protein synthesis but in contrast they display delayed activation kinetics (Tullai et al. 2007). Whilst these different classes of gene responses have been recognised for a long time the mechanisms underlying these differences in gene response are yet to be fully explored.

1.1.1 Immune cell differentiation

During the process of haematopoiesis, cells of the immune system acquire attributes which underwrite their specific function. The progressive loss of pluripotency and the process of lineage commitment occur in association with distinct changes in gene expression and cell surface markers. This process may be triggered and/or modified by a number of cues including both extracellular factors such as the cytokine environment and interactions with surrounding cells as well as intrinsic mechanisms like transcription factor up regulation and availability, in addition to epigenetic determinants (Cedar & Bergman 2011; Georgopoulos, Moore & Derfler 1992; Laiosa, Stadtfeld & Graf 2006; Rieger et al. 2009).

The haematopoietic system gives rise to a number of divergent mature immune cell types from a common hematopoietic stem cell (HSC). HSCs were first identified as $Thy^{lo}Lin^{-}Sca\text{-}1^{+}$ cells isolated from the bone marrow (Spangrude, Heimfeld & Weissman 1988). HSCs give rise to progeny that progressively lose self-renewal capacity, producing a variety of lineage committed progenitor cells and eventually differentiate into mature cell types with highly specialised roles (Akashi et al. 2000; Kawamoto, Ohmura & Katsura 1998; Lacaud, Carlsson & Keller 1998; Spangrude, Heimfeld & Weissman 1988). Haematopoiesis is highly complex. Prevailing early interpretations suggest that the initial step of differentiation involves the commitment to either the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP) (Akashi et al. 2000; Kondo, Weissman & Akashi 1997). Although another

progenitor population is reported to be lymphoid primed multipotent progenitors (CMPP) which precede the CLP cells and whilst committed to the lymphoid lineage retain the ability to give rise to granulocyte macrophage progenitors (GMP) (Cedar & Bergman 2011). Subsequent commitment of common myeloid progenitors to either the megakaryocyte/erythrocyte or granulocyte/macrophage lineages occur via mutually exclusive events (Akashi et al. 2000). The lymphoid lineage gives rise to both B cells and the T cell subsets which undergo further cell fate decisions (Yang, Bell & Bhandoola 2010). It is now recognised that lineage restriction is not necessarily as rigid as first thought, with more opportunity for cross-over between the divergent myeloid and lymphoid lineages as transcriptional programs are extensively shared and extend across lineage-potential boundaries (Laurenti et al. 2013). Myeloid potential persists even as lymphoid progenitors are branching towards T and B cells (Wada et al. 2008).

Mature cell types have highly specialised functions and a coordinated response between the different cell types is required for immune homeostasis. The two main cell types examined in this thesis are the CD4 positive (CD4+) T cells, also referred to as helper T cells, of the lymphoid lineage and macrophage cells of the myeloid lineage. Despite early divergence in the haematopoietic pathway and functionally distinct roles, CD4+ T lymphocytes and macrophages must coordinate and initiate immune responses in a cooperative manner. CD4+ T cells aid in cellular immunity by activating other T cells and macrophages as well as contributing to humeral immunity by triggering antibody production in B cells (Abbas, Murphy & Sher 1996). Macrophage cells are an integral part of the immune system not only recognising and phagocytosing foreign particles and malignant tumour cells but also play an essential role in processing foreign antigen for presentation to T cells (Gordon 1995; Gordon & Taylor 2005). As such, cells of these two highly divergent lineages retain the ability to induce the transcription of a number of shared immune modulating genes.

1.1.2 Transcription factor regulation

Gene expression patterns are largely governed by the availability and activation of transcription factors. Transcription factors assemble at specific DNA regulatory regions in association with other transcription factors and DNA binding proteins to form multi protein-DNA complexes that interact with RNA polymerase to control gene transcription (Kadonaga 1998). Several families of transcription factors have emerged as key regulators of immune cell differentiation as well as immune responses. Whilst some are lineage specific and differentially regulated themselves, such as Foxp3 in T regulatory (Treg) cells (Wei et al. 2009), others are expressed in most cells of the immune system. These include the Nuclear Factor of Activated T cells (NFAT) and Nuclear Factor – kappa B (NF-κB) families (Ghosh, May & Kopp 1998; Naito et al. 2011; Rao, Luo & Hogan 1997). Transcription factors respond to cell signalling events and once activated and in the nucleus they are able to recognise and associate with specific DNA sequences. The combination of transcriptional activators or repressors present determines if gene transcription occurs. Some transcription factors, such as the ubiquitous Specificity Protein 1 (SP1), are found predominately within the nucleus (Briggs et al. 1986). Others, including NF-κB are held in an inactive state in the cytoplasm and require the correct signalling before translocation to the nucleus and subsequent transcription factor-DNA interaction can occur. In addition, the abundance of a transcription factor within the cell can be further modified to control gene expression. Increased transcription and translation of transcription factor proteins occurs in response to cellular activation events as has been reported for both the NFAT and NF-κB family proteins (Rao et al. 2003; Shaw et al. 1988).

1.1.3 NF-κB transcription factors

The NF-κB transcription factors were first extensively characterised in B cell development controlling Igκ gene transcription (Pastinen & Hudson 2004; Sen & Baltimore 1986; Singh et al. 1986). It has since been shown that the NF-κB

transcription factors are not restricted to the B cell lineage and in fact are now recognised as central mediators of a broad range of cellular processes, particularly in both the innate and adaptive immune response (Baeuerle & Henkel 1994; Ghosh, May & Kopp 1998; Hayden & Ghosh 2008). In addition, the role of NF-κB is not limited to the immune system, having been documented as regulators in most cell types in the body (Ghosh, May & Kopp 1998).

The five NF-κB family members; NF-κB1 (p105/p50), NF-κB2 (p100/p52), Rel-A (p65), Rel-B and c-Rel share a conserved rel homology domain (RHD) which not only interacts with the inhibitory IκB family members but also enables DNA binding as well as the formation of dimers between NF-κB family members (Blank, Kourilsky & Israel 1992; Ghosh et al. 1995; Ghosh, May & Kopp 1998). Active NF-κB transcription factors are able to regulate target gene transcription through DNA binding as both homo and heterodimers. The capacity of different NF-κB dimers to regulate unique sets of genes introduces a significant level of selectivity into the NF-κB activation pathway (Smale 2012). The C-terminal domains are less conserved across the different NF-κB family members. c-Rel along with Rel-A and Rel-B harbour transactivation domains within the C-terminal region (Ballard et al. 1992; Ryseck et al. 1992; Schmitz et al. 1994). NF-κB1 and NF-κB2 on the other hand contain inhibitory domains and differ from the other NF-κB members as they are synthesised as larger precursor proteins (p105 and p100). The p105 and p100 precursor proteins are retained in the cytoplasm and are subject to ubiquitin/proteasome mediated processing prior to generation of the mature NF-κB subunits p50 and p52, which are able to translocate to the nucleus and interact with DNA (Fan & Maniatis 1991; Thanos & Maniatis 1995). Following nuclear translocation, the capacity of NF-κB proteins to mediate transcription events may be further modulated by post-translational modifications, including phosphorylation (Hayden & Ghosh 2008; Oeckinghaus, Hayden & Ghosh 2011). These nuclear modifications largely determine the strength and duration of the NF-κB transcriptional response (Chen & Greene 2004).

The NF-κB family of transcription factors are ubiquitously expressed with the exception of c-Rel which is predominately expressed in cells of the haematopoietic system, including both the myeloid and lymphoid lineages (Brownell et al. 1987; Carrasco, Weih & Bravo 1994; Weih, Carrasco & Bravo 1994). The importance of c-Rel in immune regulation is further emphasised by the finding that c-Rel knockout mice although viable, are immune deficient with impaired function of both macrophages and lymphocytes (Gerondakis et al. 1996; Kontgen et al. 1995).

1.1.4 Cell signalling events leading to NF-κB activation

Mature cell types express different cell surface receptors and utilise different intracellular signalling cascades to enable the orchestration of targeted responses to specific stimuli. The signalling cascade initiated by immune activation largely determines which transcription factors are activated and available in the nucleus to direct gene expression. NF-κB proteins can be activated in both T cells and macrophages, although via different signalling pathways, as depicted in Figure 1.1.

T cell activation requires two signals; binding of the T cell receptor (TCR) by MHC molecules and antigen alongside co-stimulation of molecules such as CD28 by the antigen presenting cell to help sustain and amplify TCR signalling (Acuto & Cantrell 2000; Acuto & Michel 2003). Activation via the TCR initiates an intracellular cascade of downstream signalling events involving activation of protein kinase and calcium signalling pathways. These include tyrosine and serine/threonine kinases and phosphatases, protein kinase C (PKC) and calcineurin (Lim et al. 2013). PKC activation results in nuclear translocation of a range of transcriptional activators, including the NF-κB transcription factors. Ultimately the signalling cascade initiates gene expression responses required for proliferation and differentiation of cells to mount an immune response (Acuto & Cantrell 2000).

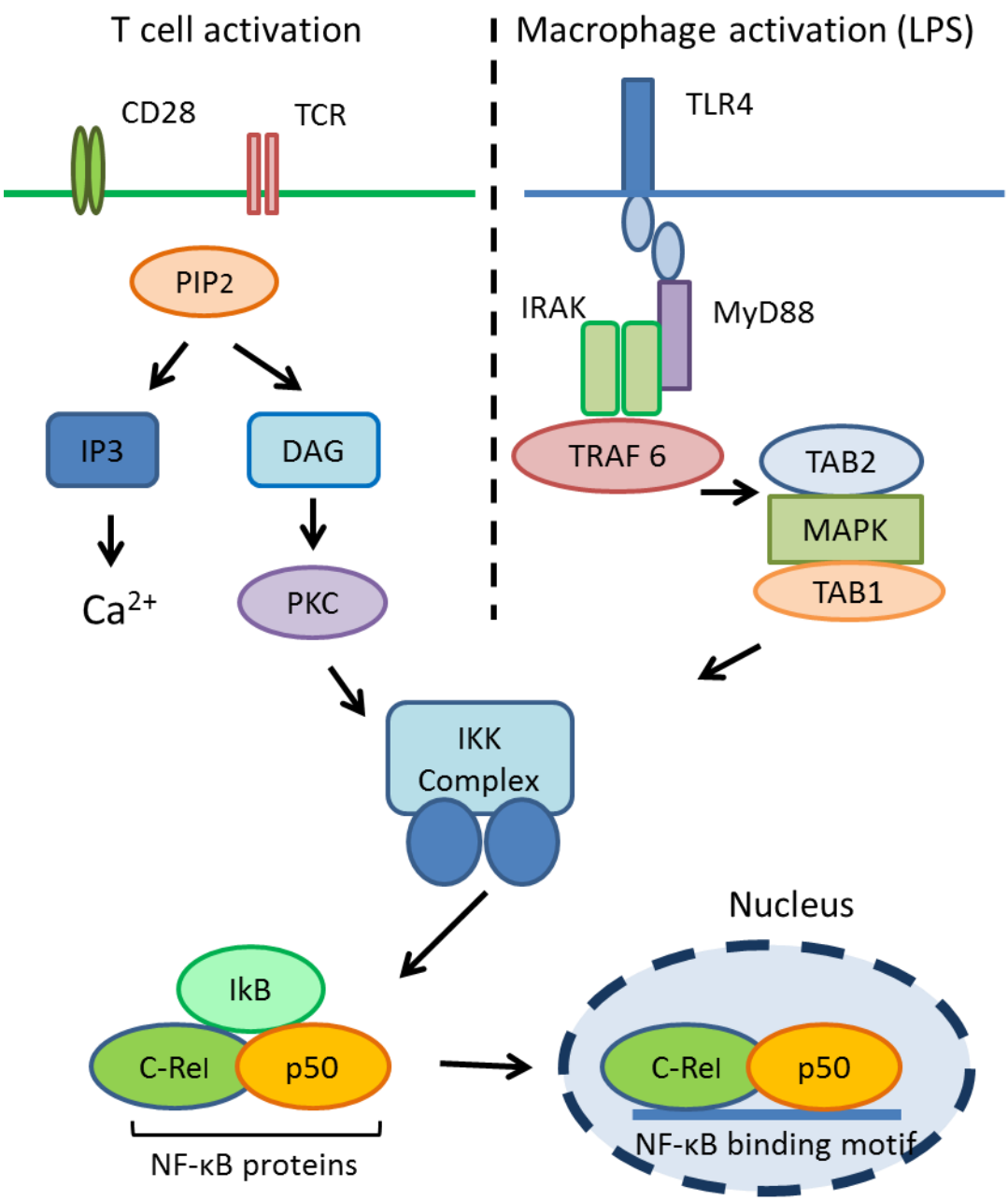

Figure 1.1: Overview of the activation of NF-κB transcription factors in stimulated T cells and Macrophages. In T cells, activation via the T cell receptor triggers activation of protein kinase C (PKC) via a series of phosphorylation events. Subsequently the IκB kinase (IKK) complex is activated, triggering the degradation of the inhibitory IκB, enabling translocation of NF-κB proteins into the nucleus. In macrophage cells stimulated with LPS, toll like receptor 4 (TLR4) is activated and myeloid differentiation primary-response protein 88 (MyD88) associates. MyD88 recruits and activates the IL-1R associated kinases (IRAK) which initiates a series of phosphorylation events leading to activation of IKK and translocation of NF-κB. For a full list of abbreviations refer to the abbreviations section on page viii.

Macrophages are heterogeneous cells varying significantly in phenotype and functionality with localisation (Gordon & Taylor 2005). As a crucial component of the innate immune system, macrophages recognise and respond to conserved, pathogen associated molecular patterns (PAMPs) through interaction with members of the toll like receptor (TLR) family. One such potent activator of macrophage immune responses is lipopolysaccharide (LPS), a major component of the bacterial outer membrane of gram-negative bacteria (Palsson-McDermott & O'Neill 2004). LPS is bound to LPS-binding proteins and recognised by toll like receptor 4 (TLR4) which, upon activation, triggers two waves of signalling. The first, MyD88 dependent signalling pathway activates early NF-κB responses via MyD88 and Mal, whilst the second MyD88 independent pathway also activates late NF-κB responses (Palsson-McDermott & O'Neill 2004). Analysis of NF-κB subunit binding through a combination of ChIP and microarray analysis in human monocyte cells found that NF-κB family transcription factors bound 157 genes in unstimulated cells and that in response to LPS stimulation this increased to 326 genes (Schreiber et al. 2006).

1.1.5 NF-κB activation pathways

Divergence in the NF-κB activation signalling pathway is one of the mechanisms by which NF-κB activation is able to influence gene expression in a spectrum of biological functions. Two distinct signalling pathways have been described extensively, termed the canonical and non-canonical pathways (Karin & Ben-Neriah 2000). Whilst general divergences between the pathways are well accepted, many of the mechanisms underlying pathway regulation remain elusive.

The canonical pathway of activation is dependent on the Inhibitor of Nuclear Factor Kappa-B Kinase Subunit beta (IKKβ) and NF-κB Essential Modulator (NEMO) subunits of the IκB kinase (IKK) complex, as defined by Oeckinghaus, Hayden and

colleagues (2011). This pathway is activated in response to most physiological activators of NF-κB signalling and as such, may be activated in response to T cell activation and macrophage TLR signalling. Pathway activation ultimately results in degradation of the classical IκB inhibitor proteins and nuclear translocation of pre-existing NF-κB dimers due to the unmasking of the nuclear translocation sequence (Beg et al. 1992). IκB degradation is mediated through the activation of the IKK complex containing the two catalytically active kinases IKKα and IKKβ and the regulatory subunit, NEMO (Yamaoka et al. 1998). The recruitment and activation of the IKK complex is itself reliant on the assembly of appropriate adaptor protein complexes.

NF-κB1 has a twofold influence on NF-κB activation. In the unprocessed p105 form it is able to associate with other NF-κB subunits tethering them in the cytoplasm. In the processed form p50 is able to translocate to the nucleus and exert gene regulatory function (Rice, Mackichan & Israel 1992). The degradation of p105 occurs by a separate pathway and is dependent upon signal-dependent proteolysis. Protein degradation releases p50 homodimers as well as p50 containing heterodimers such as p50-c-Rel. This process is mediated by the same canonical pathway, as it requires the same IKK complex components (Heissmeyer et al. 1999).

The non-canonical or alternative pathway describes the activation of p100 into the mature p52 form of NF-κB2. This pathway typically regulates p52-Rel-B dimers. The non-canonical pathway is activated independently of the NEMO and IKKβ components of the IKK complex with IκB phosphorylation mediated by IKKα proteins (Oeckinghaus, Hayden & Ghosh 2011). Activation of IKKα phosphorylation activity is dependent on NF-κB inducing kinase (NIK) which itself is subject to intricate regulation via members of the TRAF family of proteins (Adhikari, Xu & Chen 2007; Oeckinghaus, Hayden & Ghosh 2011).

It has been proposed that the NF-κB family of transcription factors exert two waves of activation following appropriate cell signalling events (Saccani, Pantano & Natoli 2001). NF-κB proteins enter the nucleus rapidly following appropriate activation

signals and a number of NF-κB response genes are able to respond by increasing transcription immediately. The second round of gene activation occurs up to 90 minutes post NF-κB translocation (Rao et al. 2003; Saccani, Pantano & Natoli 2001). Different NF-κB family members have been shown to translocate to the nucleus with different kinetics. Translocation kinetics are cell type dependent and often reflective of their relative abundance in the cytoplasm prior to stimulation. For example in T cells, Rel-A which is expressed at higher basal levels in the cytoplasm rapidly increases in the nucleus within 30 minutes of immune stimuli. A second round of translocation is observed as increased synthesis of new Rel-A protein replenishes cytoplasmic Rel-A (Brettingham-Moore 2006). In contrast c-Rel has relatively low basal cytoplasmic levels and an increase in nuclear c-Rel is not observed in T cells until after 2 hours of immune stimuli (Brettingham-Moore 2006; Rao et al. 2003).

Rather than the two rounds of gene activation being attributed to the two waves of NF-κB translocation, this is thought to be due to the differing activation requirements of the genes themselves, highlighting that gene activation is a highly regulated process involving multiple control points, as depicted in Figure 1.2 (Saccani, Pantano & Natoli 2001). This may be due to post-translational modification of the NF-κB proteins themselves following translocation or the delay in synthesis of other activation requirements, such as co-activators or repressors. Transcription may also require the interaction of chromatin modifiers or remodelling complexes to modify the chromatin structure at NF-κB target sites prior to assembly of the complete transcription initiation complex (Natoli 2009).

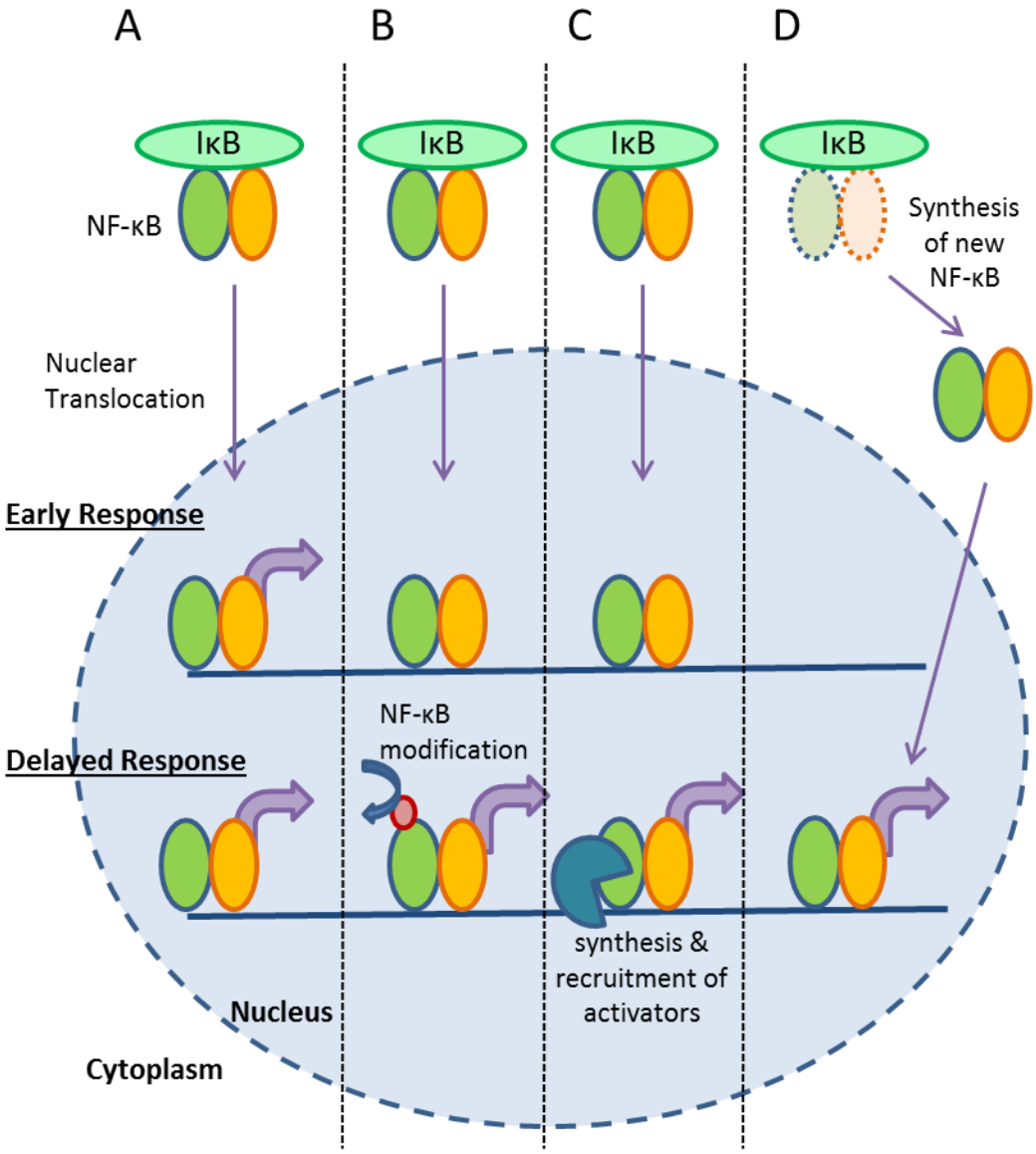

Figure 1.2: NF-κB activation checkpoints. (A) Following activation signals IκB is degraded and cytoplasmic NF-κB translocates to the nucleus resulting in increased gene transcription (early response). (B) NF-κB translocates to the nucleus and undergoes modification prior to gene transcription (delayed response). (C) NF-κB translocates to the nucleus, gene activation is delayed due to the synthesis and recruitment of additional activators such as transcription factors and chromatin remodelling complexes (delayed response). (D) Insufficient basal cytoplasmic NF-κB is present, and following activation new NF-κB proteins are synthesised before nuclear localisation can occur (delayed response).

1.2 Chromatin architecture and immune gene regulation

As immune responses are transient and involve the coordinated activity of a number of immune cell types, the majority of immune response genes are inducible and many are cell-type specific. Inducible genes are usually maintained in a repressed state with minimal or no basal gene transcription. However, in specific immune cell types, immune genes are primed to enable rapid increases in gene expression in response to appropriate activation signals. Post initiation, a transient period of high level transcription is seen before returning to basal levels as the immune response progresses (Holloway, Rao & Shannon 2002). The role of chromatin in mediating these responses is now being unravelled through the study of chromatin structure at single gene loci in response to activation signals, as well as through genome-wide analysis of chromatin structure. Such studies are revealing how nucleosome positioning, histone modification and histone variant composition contribute to cell-type and signal-specific gene expression patterns.

Within each eukaryotic cell, DNA is packaged into a highly dynamic chromatin structure. Chromatin is a nucleoprotein complex in which the DNA double helix is integrated into regularly spaced nucleosomes, the basic structural unit of chromatin (Kornberg 1974). Each nucleosome consists of an octamer of core histone proteins, containing two each of H2A, H2B, H3 and H4 (Kornberg & Lorch 1999). Histone chaperone proteins deposit H3/H4 tetramers on newly replicated DNA. A second chaperone protein then adds H2A/H2B dimers and modifies the completion of nucleosome assembly (Lorch, Maier-Davis & Kornberg 2006). This nucleosome structure is then further compacted into a higher order 30nm chromatin fibre and ultimately assembled into the chromosome (Tremethick 2007). Histone 1 (H1) proteins are linker histones structurally unrelated to the core histones, which occupy approximately 20bp within the linker regions of DNA, located in between nucleosomes. H1 proteins have an essential role in mediating chromatin condensation and stability (McBryant, Adams & Hansen 2006). While the assembly of DNA into chromatin is

essential for the ordered storage of large amounts of genetic information within the cell nucleus, it is clear that chromatin also provides an additional layer of regulatory information superimposed upon the DNA sequence (Gardner, Allis & Strahl 2011). This information may be generated by the precise positioning of nucleosomes, chemical modification and physical remodelling of the histones, and also by the incorporation of variant histone proteins.

Two distinct regions of chromatin have been identified within the cell nucleus termed heterochromatin and euchromatin (Cheutin et al. 2003; Tamaru 2010). Heterochromatin regions are typically regions where the chromatin fibre is highly condensed and are usually associated with transcriptionally silent genes. Euchromatin on the other hand is less condensed and hence allows greater accessibility of the underlying DNA to gene transcription initiation complexes. The maintenance of euchromatin and heterochromatin regions is highly complex and rather than being maintained as a static structure, it allows plasticity between the two states and visible changes in the two compartments are seen in response to stimuli (Tamaru 2010; Zhao et al. 1998). It now appears that there are many intermittent chromatin states that may be modified by histone variants, histone modifications and chromatin remodelling complexes (Luger, Dechassa & Tremethick 2012).

1.2.1 Nucleosome positioning and changes in nucleosome occupancy

The chromatin structure established at the regulatory regions of immune genes partially dictates the response of the gene to stimuli and hence contributes to the effectiveness of an immune response. During the process of immune cell differentiation, lineage specific chromatin structure is established along with lineage specific gene expression patterns. Variation in chromatin structure between different cell lineages may be visualised as the appearance of lineage specific DNase hypersensitive (DH) sites (Cockerill 2011; Hawwari et al. 2002). DH sites represent regions of DNA that are more sensitive to nucleases such as DNase and MNase. Such sites have long been

appreciated to coincide with regulatory elements of genes. Further, the appearance of DH sites has been well documented to coincide with the activation of inducible genes. Inducible DH sites typically represent regions of chromatin which have been remodelled to be more accessible to transcription factors and regulatory molecules (Cockerill 2004, 2011; Takemoto et al. 2000). Changes in gene transcription are commonly associated with changes in chromatin structure and inducible DH sites have been described at the regulatory regions of a number of different cytokine genes in immune cells in response to appropriate activation signals. Data from both single gene and genome-wide studies suggest that the promoter region of many inducible genes is encompassed within a nucleosome, and that nucleosome rearrangement or disassembly is required to enable the transcription machinery to access the gene promoter and facilitate gene activation (Workman 2006). At a single gene level, such chromatin changes have been well described during activation of many inducible genes. The *Granulocyte Macrophage – Colony Stimulating Factor* (*GM-CSF*) gene and closely linked *Interleukin 3* (*IL-3*) gene have been well characterised by DH mapping in T cells in order to identify key regulatory elements (Cockerill 2004). Both genes appear to be regulated by proximal promoters located immediately upstream of the transcriptional start site in addition to three enhancers that are identifiable by the induction of DH sites in response to stimulation (Cockerill et al. 1993). Similarly at the *interleukin 2* (*IL-2*) gene, DH sites appear in both the proximal and distal regulatory regions upon stimulation (Ward et al. 1998). Further studies revealed that inducible DH sites at the *GM-CSF* and *IL-2* promoters result from an increase in accessibility of the promoters, due to the depletion of histones from regions of the promoter corresponding to one (*GM-CSF*) or two (*IL-2*) nucleosomes, in response to activation signals (Brettingham-Moore et al. 2008; Chen et al. 2005; Holloway et al. 2003; Rao, Procko & Shannon 2001). Likewise, MNase1 analysis of the *IL-12 p40* promoter has demonstrated that selective remodelling of a single positioned nucleosome covering the promoter accompanies its activation in macrophages (Weinmann, Plevy & Smale 1999).

Genome-wide analysis of histone occupancy and positioning suggest that the remodelling events observed at these individual cytokine promoters are a relatively

common feature of inducible genes. Genome-wide maps of nucleosome positioning in both resting and activated CD4+ T cells have recently been generated using direct sequencing (Schones et al. 2008), and these data suggest that nucleosomes are highly phased relative to the transcriptional start site (TSS) of expressed genes, but that phasing is not seen at unexpressed genes (Schones et al. 2008). Nucleosome phasing appears to be influenced by surrounding histone features such as the incorporation of histone variants and the presence of poised or elongating Pol II at the TSS (Schones et al. 2008). Furthermore, data from this study suggest that many inducible genes are in a permissive basal state in terms of nucleosome positioning but that significant nucleosome reorganisation occurs following TCR stimulation, with a decrease in histone density preferentially occurring at the promoters of activated genes. Remodelling appears to alter the chromatin surrounding the promoters of inducible genes so that they are more accessible to the transcription machinery (Schones et al. 2008).

1.2.2 Histone modification signatures

While changes in nucleosome occupancy are clearly important in regulating gene expression, the modification state of these nucleosomes also influences gene activity. Each of the core histone proteins consists of an inner globular domain into which the DNA is embedded and an N terminal tail region of between 14 and 28 amino acids, which extends beyond the nucleosome complex (McBryant, Adams & Hansen 2006). The histone proteins are subject to a range of modifications, occurring primarily on the histone tails. There have now been more than a dozen forms of histone modification described, the most extensively studied being acetylation and methylation (Barski et al. 2007; Gardner, Allis & Strahl 2011; Kouzarides 2007; Tan et al. 2011).

1.2.2.1 Histone Acetylation

Acetylation of histones on lysine residues is strongly correlated with active gene transcription (Strahl & Allis 2000), although the particular mechanisms by which histone acetylation facilitates gene expression have yet to be fully elucidated. Initially the correlation between histone acetylation and increased gene transcription was attributed to acetylation neutralising the positive charge of lysine residues within the histone. This is thought to reduce the affinity between the histone tails and the negatively charged DNA on surrounding nucleosomes, thus loosening the overall chromatin structure and enabling transcription from the underlying DNA (Grunstein 1997; Shogren-Knaak et al. 2006). Acetylation marks may also serve as recognition sites for bromodomain containing protein complexes. Bromodomains recognise acetylated lysine residues and are contained within a number of chromatin modifying complexes and transcriptional regulators such as histone acetyltrasferases, histone methyltransferases and chromatin remodelling complexes. Thus histone acetylation may mark regions of chromatin for downstream modification and subsequent chromatin remodelling events (de la Cruz et al. 2005).

1.2.2.2 Histone Methylation

Histone methylation can occur on either arginine residues, which may be mono- or di-methylated, or lysine residues which may be mono-, di- or tri- methylated (Rice et al. 2003). Histone methylation is somewhat more complicated than acetylation as it is associated with both activation and repression of gene expression. The effect of methylation is determined by the precise location of the amino acid residue on which the methylation occurs, and the number of methylation groups added (Barski et al. 2007). This observation led to the proposal of the so-called “histone code” hypothesis, in which histone tail modifications act as receptors to be recognised and bound by other proteins which further modify chromatin structure (Gardner, Allis & Strahl 2011; Strahl & Allis 2000). For example, histone 3 lysine 27 (H3K27) di- and tri-methylation is well

documented as being a repressive histone modification required for mediating gene silencing. H3K27me2/3 is recognised and bound by polycomb repressor complex 2 (PCRC2), a methyltransferase which further catalyses the addition of repressive histone modifications, thus maintaining gene silencing throughout processes such as DNA replication (Xu et al. 2010).

Histone H3 methylation at lysine 4 (H3K4) is associated with euchromatic regions. Tri-methylation at this position generally localises at the 5' gene regulatory regions, near to the transcriptional start site. This methylation mark correlates with actively transcribed genes (Bernstein et al. 2005; Santos-Rosa et al. 2002). A number of proteins have been found to recognise and interact with H3K4me3 including several chromatin remodelling proteins. The yeast homologue of the human ISWI chromatin remodelling enzyme, Isw1p has been shown to mediate chromatin remodelling events to make the regulatory region of genes marked by H3K4me3 more permissible to transcription initiation (Santos-Rosa et al. 2003). In addition, the chromodomain-helicase-DNA-binding protein 1 (CHD1) has also been shown to associate with H3K4me3 and recruit factors required for efficient RNA transcription post initiation, thereby facilitating transcription through positioned nucleosomes (Sims et al. 2007). These studies suggest that the H3K4me3 acts as a recognition site to enable the assembly of other co-activators and transcription machinery, permitting gene transcription to occur.

1.2.3 Histone modifications in inducible immune gene expression

The functional role of many of the histone modifications remains elusive, although it is clear that such modifications can have a profound effect on the overall chromatin structure and function. Recent data, particularly that generated using genome-wide technologies such as ChIP-chip and ChIP-sequencing, has begun to shed new light on the distribution and role of permissive and repressive histone modifications in regulating gene expression (Barski et al. 2007; Lim et al. 2009; Roh, Cuddapah & Zhao 2005; Schones et al. 2008; Wang et al. 2008). This work has begun to define chromatin

signatures that are associated with particular gene states, providing insight into how different chromatin architectures interact with extracellular signals and regulate gene expression, as reviewed by Lim et al. (2013).

Early studies investigating histone acetylation patterns associated with the *IL-4* and *IFN-γ* genes during T cell differentiation using Chromatin Immunoprecipitation (ChIP) assays, indicated that specific histone acetylation patterns where generated at the gene loci during differentiation, establishing permissive chromatin environments (Avni et al. 2002). Differentiation of naïve T cells down the Th1 or Th2 lineage results in cells which express either *IFN-γ* or *IL-4* respectively (Mosmann & Coffman 1989), correlating with the accumulation of histone acetylation at the active locus (Wei et al. 2009). Acetylation marks are often associated with actively transcribed genes. Genome-wide analysis of histone acetylation in T cells described an association of H3K9ac/K14ac with regulatory regions, including both promoters and enhancers, with the acetylation levels correlating well with gene expression. The discovery that promoter regions are often occupied by highly acetylated histones in comparison to the rest of the genome and the finding that promoters containing unmethylated CpG islands are often marked by highly acetylated histones, led to the proposition of 'acetylation islands'. It was hypothesised that histone hyper-acetylation functions as a mechanism to prevent DNA methylation and hence is correlated with genes being maintained in an inducible state (Roh, Cuddapah & Zhao 2005). Subsequent ChIP-chip experiments in T cells revealed that inducible genes exhibit higher levels of acetylation as well as permissive methylation marks when compared to non-expressive genes, suggesting that permissive histone modifications may act to prime genes for transcriptional response upon activation (Lim et al. 2009). Furthermore, it was found that early response genes have significantly greater acetylation than delayed response genes, suggesting that the histone modifications may determine not only the ability of a gene to respond to activating signals, but also the kinetics of the gene response (Hargreaves, Horng & Medzhitov 2009). Histone acetylation has generally been associated with gene transcription and this has largely been confirmed by genome-wide studies. One such genome-wide study profiled 18 different acetylated lysine residues on the four core

histones and demonstrated that all were associated with gene transcription (Wang et al. 2008). However there are also reports of particular histone acetylation marks being associated with gene silencing. For example, H4K12ac has been demonstrated to have a role in transcriptional silencing in heterochromatin in yeast (Braunstein et al. 1996).

In contrast to histone acetylation, which is largely associated with gene transcription, histone methylation is associated with either activation or repression depending on the particular modification. Genome-wide analysis of the distribution of H3K27me3 and H3K9 methylation suggest these are repressive histone modifications as they are more prevalent at silenced genes compared to active promoters (Barski et al. 2007). The association of these marks with a repressive role was confirmed in a further study, along with H3K27me2 and H4K20me3 (Wang et al. 2008). However, in addition to marking promoters of inactive genes these modifications are also present in intergenic regions (Barski et al. 2007; Roh et al. 2006). In contrast, H3K4 methylation appears to be a permissive methylation mark, with H3K4me3 enriched at the TSS of active genes, and H3K4me1/2 enriched across the transcribed regions of active genes (Barski et al. 2007). Similarly, H3K36me3 and H3K79 methylation are associated with actively transcribed genes, although H3K36me3 is enriched at the 3' end, while H3K79 methylation is enriched at the 5' end of these genes. Furthermore, while di- and tri-methylation of H3K9, H3K27 and H4K20 are associated with gene repression the mono-methylation of these residues is enriched at active genes (Barski et al. 2007).

1.2.4 Bivalent histone modifications

Specific histone modifications are clearly associated with gene activation or gene repression. However, genome wide studies have also identified genes which carry so-called 'bivalent' marks and are marked by both active (H3K4me3) and repressive (H3K27me3) histone modifications (Azuara et al. 2006; Bernstein et al. 2006; Mikkelsen et al. 2007). Bivalent modifications are thought to enable a gene to be held in a non-transcribed state by the repressive markers, while it is also primed to respond

to appropriate signals by the permissive marks. Bivalent histone modifications were initially thought to be a unique feature of stem cells and to contribute to the pluripotency, but have subsequently been observed in differentiated cells. It has therefore been suggested that this bivalent chromatin state may be involved in regulating genes that respond to developmental and environmental signals in many cell types (Araki et al. 2009; Barski et al. 2007; Cui et al. 2009; Lim et al. 2009; Rodriguez et al. 2008).

1.2.5 Histone modification enzymes

The modification state of histones is maintained by the activity of groups of modifying enzymes. Acetylation levels are determined by the opposing activity of histone acetyl-transferases (HAT)s and histone deacetylases (HDAC)s, whilst methylation modifications are mediated by histone methyl-transferases (HMTs) and demethylases (Couture & Trievel 2006; Smith & Denu 2009). Thus the activity of modifying enzymes determines the level of permissive and repressive markers at each gene. Recent ChIP-sequencing studies in T cells have profiled the association of HATs with gene promoters (Wang et al. 2009). Combined analysis with gene expression data reveals that, as may be expected, a high level of HAT activity correlates with higher levels of acetylation, in addition to elevated gene transcription. In addition HAT binding is positively correlated with the binding of Pol II at active genes (Wang et al. 2009). Surprisingly, ChIP-sequencing revealed that HDACs are also correlated with active gene transcription, with only a small fraction associated with silent genes. These results suggest that rather than having a primary role in maintaining a silent state, the main role of HDACs may be the removal of acetyl groups added by HATs at active genes. HDACs are recruited to actively transcribed genes by interaction with elongating Pol II and may therefore play an important role in resetting chromatin following remodelling (Wang et al. 2009). Examining the binding of HATs and HDACs to transcriptionally silent genes reveals that these genes are also subject to constant acetylation and deacetylation cycles mediated by the transient and unstable binding of

HATs and HDACs. Thus like 'bivalent' chromatin marks, the activity of HATs and HDACs at repressed genes primes these genes for future activation (Wang et al. 2009). Furthermore, activity of HATs and HDACs at 'primed' genes appears to be further modulated by the presence of other chromatin modifying factors including permissive H3K4 methylation and the histone variant protein H2A.Z (Wang et al. 2009).

1.2.6 Chromatin remodelling complexes

In addition to the proteins that modify histones, a group of chromatin remodelling complexes (CRCs) have been described. CRCs utilise the energy released by ATP hydrolysis to physically alter chromatin structure through changes in the association between histone proteins and the DNA double helix. CRC activity may result in changes in nucleosome conformation, position, composition and occupancy and are often associated with specific histone modifications (Li, Carey & Workman 2007). These mechanisms provide another layer of complexity to gene regulation by dictating the accessibility of regulatory regions of DNA to transcription factors. The sliding and partial or full disassembly of the core nucleosome proteins by chromatin remodelling complexes may disrupt the DNA/histone protein interaction, allowing transcription factors to access and bind DNA regulatory regions and initiate transcription. Likewise re-assembly of the nucleosome complex may conclude gene transcription (Kornberg & Lorch 2007). It has been proposed that remodelling enzymes may be targeted to specific DNA regions through interaction with transcription factors, non-coding RNA, specific histone modifications and/or DNA damage (de la Serna, Ohkawa & Imbalzano 2006). CRCs may be classified into subfamilies by the sequence homology of their ATPase subunit. These families are called SWI/SNF (SWItch/Sucrose Non-Fermentable), ISWI (Imitation SWItch), CHD1 (chromodomain helicase DNA binding protein) and INO80 (INOsitol requiring) (Lusser & Kadonaga 2003). CRC sub-families have both complementary and opposing roles depending on the cell type and chromatin environment.

CRCs frequently mediate the changes in chromatin accessibility required for transcriptional activation of inducible genes. Utilising ATP-hydrolysis, CRCs reposition, assemble or dismantle nucleosome complexes from the regulatory regions of genes (Lusser & Kadonaga 2003; Saha, Wittmeyer & Cairns 2006). However chromatin remodelling is not required for activation of all genes. Genes that respond rapidly to immune stimuli often exhibit high sensitivity to nucleases at regulatory regions prior to stimulation. Further analysis revealed that this is due to low nucleosome occupancy at nuclease susceptible promoters (Ramirez-Carrozzi et al. 2009). Genes that have low basal nucleosome occupancy are therefore proposed to increase gene expression independent of chromatin remodelling as the promoter is subject to less nucleosome interference with transcription factor association.

Lymphocyte activation is accompanied by visible changes in the chromatin architecture which are associated with the stable association of SWI/SNF complexes with the nucleus/chromatin within 10 minutes of cell stimulation (Zhao et al. 1998). Genome-wide analysis of BRG1 (an ATPase component of the SWI/SNF complex) binding in non-stimulated and stimulated T cells in conjunction with mRNA gene expression analysis reveals that BRG1 is important for the response to cell activating signals and that BRG1 binding is positively correlated with gene transcription (De et al. 2011). Knockout of the BRG1 gene is embryonically lethal during the implantation stage however, conditional knockdown in fibroblast cells does not affect survival in culture suggesting that BRG1 is required for development but not general cell viability (Bultman et al. 2000). Furthermore, conditional knockdown of BRG1 has demonstrated BRG1 is required for multiple stages of T cell development as BRG1 is required for effective intracellular signalling events (Chi et al. 2003; Gebuhr et al. 2003). Knockdown of the BRG1/BRM complex in murine macrophages has shown that a subset of inducible genes require SWI/SNF for activation in macrophages also (Ramirez-Carrozzi et al. 2009). The recruitment of BRG1 to SWI/SNF dependent genes is reported to be mediated by the presence of permissive histone acetylation marks (De et al. 2011). In this case, for chromatin remodelling to occur via the recruitment of

BRG1 a specific chromatin environment must be established at the gene prior to recruitment.

SNF2H is an ISWI chromatin remodelling complex which is also abundantly expressed in immune T cells. Knockdown of SNF2H revealed that SNF2H has a regulatory role at a number of cytokine genes, typically repressing gene transcription, but at some cytokine genes such as *IL-3*, increasing transcription (Precht, Wurster & Pazin 2010). SNF2H appears to mediate changes in chromatin structure by directly binding in association with its partners, including ACF1, to regulatory sites. However cell stimulation appears to have minimal influence on SNF2H binding, which remains comparatively uniform across cytokine loci. Knockdown experiments have demonstrated that SNF2H is crucial in mediating development and as there is little evidence of dynamic recruitment of the remodeller, it appears that SNF2H association with chromatin at cytokine genes may be initiated during differentiation (Precht, Wurster & Pazin 2010).

Mi2/NuRD complexes interact with DNA-binding proteins such as Ikaros which are implicated in repression, as well as HDACs and have been shown to contribute to transcriptional repression. However the complex has also been implicated in transcriptional activation (Ramirez-Carrozzi et al. 2006). In macrophage cells, the Mi2β/NuRD complex associates with pro-inflammatory cytokine genes in a similar way to the BRG1 SWI/SNF remodeller. Knockdown of Mi2β revealed that Mi2β acts as an antagonist of gene transcription, moderating the response of late primary and early secondary response genes. When BRG1 is depleted the association of Mi2β with cytokine genes is also depleted, suggesting that Mi2β is dependent on BRG1 for recruitment to regulatory regions to occur (Ramirez-Carrozzi et al. 2006).

1.2.7 Histone variants

Chromatin structure can also be modified by the incorporation of variants of the canonical core histone proteins. Variants are so named as they are variations of the major histone proteins. They may vary in only a single amino acid or exhibit more significant alterations in composition. The histone variant H2A.Z is a replacement histone for H2A, the sequence of which varies significantly from that of canonical H2A (Bonenfant et al. 2006). H2A.Z appears to have a key role in chromatin structure determination (Creyghton et al. 2008). In human CD4+ T cells, H2A.Z is associated with promoter regions both up and downstream of transcription start sites. Integration of the H2A.Z histone appears to be associated with gene activity (Barski et al. 2007). H3.3 is another frequently reported histone variant, but in contrast to H2A.Z, H3.3 is highly structurally similar to H3 (Szenker, Ray-Gallet & Almouzni 2011). The integration of H3.3 into nucleosomes is strongly associated with gene transcription and it is thought to destabilise nucleosome structure (Jin et al. 2009; Kafer et al. 2010; Sutcliffe et al. 2009; Szenker, Ray-Gallet & Almouzni 2011). Not only are histone variants associated with regulating gene expression, they have also been implicated in DNA repair and X inactivation. Variant histone proteins have also been shown to be enriched at target genes in embryonic stem cells and appear to be necessary for lineage commitment to occur (Creyghton et al. 2008).

1.3 Immune gene activation in divergent cell types

The advent of genome-wide technologies to study chromatin architecture has provided remarkable insight into the multitude of chromatin factors which mediate gene activity, including histone modifications, activity of histone modifying enzymes and recruitment of chromatin remodelling complexes as well as the incorporation of histone variants (Araki et al. 2009; Barski et al. 2007; De et al. 2011; Ramirez-Carrozzi et al. 2009).

Such analysis has provided general "rules" for deciphering chromatin signatures associated with particular gene states, as summarised in Figure 1.3. In addition, genome-wide data is available for association of transcription factors to regions of DNA both before and after immune stimuli (Bunting et al. 2007) as well as the association of poised and recruited RNA polymerase II (Barski et al. 2009). Genome-wide studies continue to be complimented by gene-centric studies which are uncovering the key mechanisms that regulate gene expression, and how particular chromatin features interact with modifying proteins and transcription factors to impact gene activity. Despite the insights that we have gained over recent years several questions remain unanswered including how conserved the regulatory mechanisms are across different cell types.

The inducible expression of the inflammatory cytokine, Granulocyte Macrophage – Colony Stimulating Factor (GM-CSF) has been studied extensively in T cells where gene activation has been shown to require co-operative interaction with both the NF-κB transcription factor, c-Rel and the BRG1 chromatin remodelling complex (Brettingham-Moore et al. 2008; Poke et al. 2012). *GM-CSF* expression is not restricted to the T cell lineage, with expression also inducible in response to stimuli in several other cell types including macrophages (Cockerill 2004; Shannon et al. 1997). Several other inducible cytokine genes are also expressed in both T cells and macrophages which may be regulated by similar mechanisms to *GM-CSF*.

The hypothesis underlying this study is that during the process of differentiation lineage specific chromatin structure is established at inducible gene promoters and that this chromatin structure largely dictates the cooperative regulation of gene expression through interaction with transcription factors, structural proteins and chromatin remodelling enzymes.

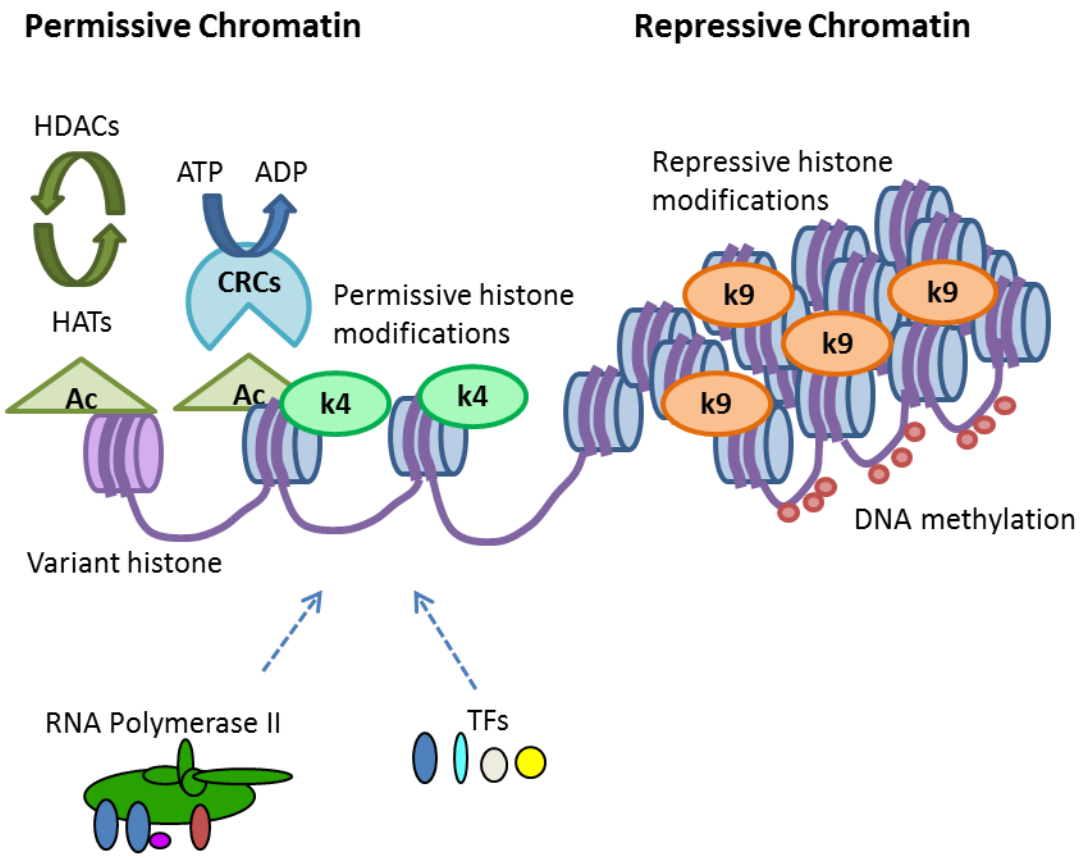

Figure 1.3: Chromatin structure and modification. DNA can be held in a permissive or repressive chromatin structure which largely dictates if gene transcription takes place. This is achieved through the actions of chromatin modifying and remodelling complexes. Chromatin remodelling complexes (CRCs) can restructure, reposition and evict histone octamers using ATP to regulate access to DNA for the binding of transcriptional regulatory factors like transcription factors (TFs), coactivators and the basal transcription machinery including RNA polymerase. Histone tails can be modified by an extensive range of histone modifications including phosphorylation, acetylation (Ac) and methylation. Methylation may be either permissive (e.g H3K4) or repressive (e.g H3K9) depending on which tail residue is modified. This is achieved through the action of histone modifiers like histone acetylases (HATs) and histone deacetylases (HDACs), histone methylases and histone kinases. Histone variants such as H2A.Z and H3.3, flank nucleosome-free regions of actively transcribing promoters and other regulatory elements.

1.4 Research Project Aims:

The aim of this study was to examine how different cell types are able to induce transcription of the same cytokine genes utilising different mechanisms and to examine the role of c-Rel in facilitating this. The specific aims were to:

1) Characterise the different activation requirements of the *GM-CSF* gene in macrophages in comparison to T cells, and investigate the mechanisms involved in the different cell types.

2) Utilise genome-wide data to identify c-Rel dependent genes that have inducible expression in both T cells and macrophages and to determine whether c-Rel dependent genes are regulated by fundamentally different mechanisms in the two cell types.

2 Materials and Methods

2.1 Cell culture

2.1.1 Tissue culture

RAW 264.7 macrophage and EL4 T cell lines were purchased from the American Tissue Culture Collection and were cultured in Gibco® RPMI 1640 medium (Invitrogen™, U.S.A) supplemented with fetal bovine serum (10%; JRH Biosciences, Australia), penicillin (100U/mL)/streptomycin (100µg/mL, Sigma-Aldrich, U.S.A). EL4 T cells were maintained between $2x10^5$ and $1x10^6$ cells per mL with passaging every 24hrs. RAW 264.7 macrophages were subcultured every 3 days by cell scraping and re-plating at 1:4. Cells were incubated in a humidified atmosphere at 37°C with 5% CO_2.

2.1.1.1 Treatment of cultured cells

Cell lines and primary cells were treated with the following reagents as indicated in Table 2.1.

Table 2.1: Cell treatments

Reagent	Stock concentration	Treatment
lipopolysaccharide from *salmonella typhosa* (LPS, Sigma-Aldrich, U.S.A).	1mg/mL in RPMI	10µg/mL for variable times as indicated.

phorbol 12-myristate 13-acetate (PMA, Boehringer Mannheim, Australia).	1mg/mL in DMSO	20ng/mL, for variable times as indicated.
Calcium ionophore A23187 (I, Sigma-Aldrich, U.S.A).	10mM in DMSO	1µM, for variable times as indicated.
Cyclohexamide (CHX, Millipore, Germany).	100mg/mL in ethanol	10 µM for 30 minutes.
Pentoxyfylline (PTX, Sigma-Aldrich, U.S.A).	Stored as powder and made up fresh by dissolving directly in medium 1 hour prior to treatment.	6mg/mL for 30 minutes.

Unless otherwise specified EL4 T cells were stimulated using 20ng/mL phorbol 12-myristate 13-acetate (PMA, Boehringer Mannheim, Australia) and 1µM calcium ionophore (A23187; Sigma-Aldrich, U.S.A). RAW 264.7 cells were stimulated using 10ng/mL bacterial lipopolysaccharide (LPS) isolated from *Salmonella Typhosa* (Sigma-Aldrich, U.S.A).

2.1.2 Isolation of primary cells

Ethics approval was sought and gained from the University of Tasmania Animal Ethics Committee (ethics number: A0011434). Primary cells were obtained from male C57/BL6 mice and c-Rel$^{-/-}$ mice aged 6-8 week. c-Rel$^{-/-}$ mice were obtained from the lab of H.C Liou (Liou et al. 1999) and re-derived at the John Curtin School of Medical Research, Australian National University (JCSMR, ANU). Experiments were undertaken at JCSMR, ANU (ethics numbers: ASD 197 and ASD 173).

2.1.2.1 CD4+ T cells

CD4+ T cells were isolated using the MACS CD4+ T cell isolation kit (Miltenyi Biotec, Germany) as per the manufacturers' directions. Briefly, mice were euthanised by CO_2 asphyxiation and spleens were isolated. Splenocytes were dissociated by passage through a 70µM cell strainer (Becton Dickinson Biosciences, Australia). Mononuclear cells were collected utilising density gradient centrifugation through Lympholyte-M (Cedarlane, Canada) in accordance with the manufacturer's directions. Cells were then incubated with antibody-conjugated magnetic beads against CD4 (Miltenyi Biotec, Germany). CD4+ cells were selected by magnetic separation, according to the manufacturers' directions. Purified cells were maintained in Gibco® DMEM supplemented with fetal bovine serum (10%; JRH Biosciences, Australia), penicillin (100U/mL) /streptomycin (100µg/mL, Sigma-Aldrich, U.S.A).

2.1.2.2 Murine Embryonic Fibroblasts (MEFs)

Murine embryonic fibroblasts were obtained from E14 C57/BL6 embryos. Embryos were decapitated and the liver removed. Remaining embryonic tissue was finely minced using a scalpel blade and suspended in 10mL of 1 x PBS (MP Biomedicals, U.S.A) with trypsin (0.2%, Sigma-Aldrich, U.S.A). Cells were incubated in trypsin for 10 minutes at room temperature before being pelleted by centrifugation (300g for 5 minutes). Cells were resuspended in 2mL 1 x PBS (MP Biomedicals, U.S.A) and dissociated through a 70 µM cell filter (Becton Dickinson Biosciences, Australia) with excess 1X PBS (MP Biomedicals, U.S.A) before being pelleted by centrifugation (300g for 5 minutes) and re-suspended in 10mL DMEM (Invitrogen™, U.S.A) at 37°C and transferred to 25cm^2 flasks (Becton Dickinson Biosciences, Australia). Following 24 hours incubation, adherent fibroblasts were further subcultured every 3 days as cells reached approximately 90% confluence. To subculture, adherent cells were washed in 1 x PBS (MP Biomedicals, U.S.A) before 3 minute incubation in 5mL 0.05% trypsin in

PBS (Sigma-Aldrich, U.S.A) at 37°C. Flasks were tapped to ensure dissociation of adherent cells and 5mL of DMEM medium was added before cells were pelleted by centrifugation (300g for 5 minutes). Cells were resuspended in fresh DMEM (Invitrogen^{TM}, U.S.A) and re-plated 1:4.

2.1.2.3 Bone Marrow Derived Macrophages (BMDM)

Mice were euthanised by CO_2 asphyxiation. Bone marrow cells were flushed with DMEM (Invitrogen^{TM}, U.S.A) from mice tibias and femurs using a 23 gauge needle and syringe. Cells were then dissociated through a 70 µM cell filter (Becton Dickinson Biosciences, Australia) with excess 1X PBS (MP Biomedicals, U.S.A) before being pelleted by centrifugation (300g for 5 minutes) and re-suspended in 10mL DMEM at 37°C and transferred to 25cm^2 flasks (Becton Dickinson Biosciences, Australia). Cells were incubated overnight (37°C, 5% CO2) to allow fibroblast cells to adhere to the flask. Following 24 hours of incubation non-adherent cells were seeded into new flasks in fresh DMEM with murine macrophage colony stimulating factor, M-CSF (100ng/mL, Sigma-Aldrich, U.S.A) at a density of 1x10^7 cells per 25cm^2 flask and returned to the incubator for 5 days to allow differentiation into adherent monocyte/macrophage cells. Differentiation was determined by morphological changes as well as flow cytometry with anti-CD11b (Becton Dickinson Biosciences, Australia).

2.1.2.4 Flow cytometry

Successful isolation of primary cell populations was confirmed by flow cytometry using the CyAn^{TM} ADP Analyzer (Beckman Coulter, U.S.A). An aliquot of cells was washed in 1x PBS (MP Biomedical, U.S.A) and either left unstained as a control or incubated for 1 hour with appropriate antibody. Anti-CD11b-FITC (Becton Dickinson Biosciences, Australia) was used to detect monocyte/macrophage cells whereas Anti-CD4-FITC (Becton Dickinson Biosciences, Australia) was used for CD4+ T cells. Stained cells were analysed alongside an unstained control as well as both negative and

compensation control beads incubated with the same antibody. These protocols typically generate cell populations of over 90% purity.

2.2 Gene expression analysis by Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR)

2.2.1 RNA extraction

RNA was extracted from a minimum of 2.5 x10^6 cells per treatment. Pelleted cells were resuspended in TRI reagent (500µL, Sigma-Aldrich, U.S.A) and incubated at room temperature for 5 minutes. Chloroform (100µL) was added and the tube contents mixed by vigorous shaking for 15 seconds. Following incubation (10 minutes, room temperature) the tubes were pelleted by centrifugation at 12,000g for 15 minutes. The clear upper layer was transferred to a fresh 1.5mL microcentrifuge tube and isopropanol (250µL) was added and mixed gently by inversion to precipitate the RNA. The RNA was precipitated at -20°C for a minimum of 1 hour. Following precipitation RNA was pelleted by centrifugation at 12,000g for 20 minutes at 4°C. RNA pellets were washed with 700µL of 70% ethanol prior to air-drying. Dried pellets were resuspended in DEPC treated water (Sigma-Aldrich, U.S.A), quantified by spectrophotometery and RNA quality and integrity determined by agarose gel electrophoresis (2.2.2) before being stored at -80°C.

2.2.2 RNA quantification

RNA was quantified using the NanoDrop® ND- 1000 UV- vis spectrophotometer (Nanodrop® Technologies, U.S.A). Optical density was measured at an absorbance of 260nm and RNA concentration was determined (1 OD = 40µg/µL).

RNA integrity was confirmed by electrophoresis through a 1% agarose gel in 1x TAE and visualised using SYBR® safe DNA gel stain (Invitrogen Corporation, U.S.A). Molecular weight standards HindIII λ (New England Biolabs, U.S.A) and HyperLadder II (Bioline, UK) were electrophoresed alongside the RNA for sizing comparison.

2.2.3 Preparation of cDNA

Total RNA (1µg) was subjected to DNase I treatment for 30 minutes at 37°C using 1 unit of DNase I (Invitrogen™, U.S.A) in 1x First Strand Buffer (Invitrogen™, U.S.A). DNase I was then inactivated at 75°C for 5 minutes. Oligo dT (0.1nmol; Invitrogen™, U.S.A) was then added and the reaction was incubated at 70°C for 10 minutes. The sample was then subjected to reverse transcription using Superscript Reverse Transcriptase III enzyme (100 units, Invitrogen™, U.S.A) in 1x First Strand Buffer (Invitrogen™, U.S.A) supplemented with DTT (0.1M, Invitrogen™, U.S.A) and dNTPs (0.5mM, Promega Corporation, U.S.A). The reaction was incubated at 42°C for 50 minutes, following which the enzyme was inactivated at 70°C for 15 minutes. cDNA was stored at -20°C.

2.2.3.1 qPCR

cDNA was diluted to a concentration of 10ng/µL and 5µL (50ng) was used for each qPCR reaction. Gene expression was determined using the primer sets detailed in Table 2.2. Expression levels were determined from Ct values and a 5 point standard curve for each primer set and normalised to the "house-keeping" gene GAPDH. qPCR utilised HotStarTaq DNA Polymerase and SYBR Green I dye (QuantiTect® SYBR® Green master mix; Qiagen, Netherlands). A reaction volume of 25µL was used to analyse 50ng of DNA (or cDNA) in 5µL volume. Each reaction consisted of: QuantiTect® SYBR® Green master mix (12.5µL), RNase-Free water (4.5µL), forward primer (5 µM, 1.5µL), reverse primer (5µM, 1.5µL). For no template control reactions, the

volume of DNA was substituted for additional RNase-Free water. The samples were subjected to the following cycling using the Corbett Rotor- Gene™ 2000 Real-Time Cycler (Corbett Research Pty Ltd, Australia); 1X 95°C 15 minutes, 40X 94°C 15 seconds followed by 60°C for 60 seconds (acquiring to Cycling A on CH1) and a final melt analysis starting at 60°C ramping to 95°C, increasing by 1ºC each 5 seconds (acquiring to Melt A on CH1). Analysis was performed using the software provided (Corbett Research Pty Ltd, Australia).

Table 2.2: Primers for analysis of gene expression by RT-qPCR

Name	Sequence (5' to 3')	Fragment Size (bp)
m*GAPDH*	**F:** AAGTATGATGACATCAAGAAGGTGGT **R:** AGCCCAGGATGCCCTTTAGT	**67**
m*GM-CSF*	**F:** AAGGTCCTGAGGAGGATGTG **R:** GAGGTTCAGGGCTTCTTTGA	**140**
m*IL-23A*	**F**: GGTGCTTATAAAAAGCCAGACC **R**: AATAATGTGCCCCGTATCCA	**112**
m*IL-10*	**F:** GTGAAAATAAGAGCAAGGCAGTG **R:** GATCATCATGTATGCTTCTATGCAG	**128**
m*IL-6*	**F:** GTTCTCTGGGAAATCGTGGA **R**: TTTCTGCAAGTGCATCATCG	**85**
m*Tnfsf9* (Ramirez-Carrozzi et al. 2009)	**F**: GCCCCAACACTACACAACAG **R**: GCTGTGCCAGTTCAGAGTTG	**95**

m*IL-1a*	**F:** AGCAGCCTTATTTCGGGAGT **R**: GTGCAAGTGACTCAGGGTGA	**94**
m*IL-13* (Wurster & Pazin 2008)	**F**: AGACCAGACTCCCCTGTGCA **R**: TGGGTCCTGTAGATGGCATTG	**123**
m*IL-21* (Chen et al. 2010)	**F**: TCAGCTCCACAAGATGTAAAGGG **R**: GGGCCACGAGGTCAATGAT	**121**
m*IL-22*	**F**: CAACTTCCAGCAGCCATACA **R:** GTTGAGCACCTGCTTCATCA	**157**
m*IL-24*	**F:** TTAGGACCCTAGCAGGAGCA **R:** AGAACCACCCCTGTCACTTG	**163**

2.3 Western blot analysis of protein localisation

Table 2.3: Primary and secondary antibodies used for western blot analysis

Affinity	Host	Type	Dilution	Supplier (cat. No)
Anti - NFκB p65 (C-20)	Goat	Polyclonal	1 : 1000	Santa Cruz Biotechnology (sc-272X)
Anti - c-Rel (C)	Rabbit	Polyclonal	1 : 1000	Santa Cruz Biotechnology (sc-71)
Anti – Sp1	Rabbit	Polyclonal	1 : 1000	Santa Cruz

				Biotechnology (sc-59)
Anti – Histone H3	Rabbit	Polyclonal	1 : 10,000	Abcam (ab1791)
Anti – β-actin	Mouse	Monoclonal	1: 10,000	Sigma-Aldrich (A2228)
2° Anti – goat HRP	Rabbit	Polyclonal	1 : 1000	Dako (P0160)
2° Anti – rabbit HRP	Goat	Polyclonal	1 : 1000	Dako (P0448)

2.3.1 Preparation of nuclear and cytoplasmic protein extracts

Nuclear and cytoplasmic extracts were prepared from a modified method (Schreiber et al. 1989). Briefly, cells (up to $1x10^7$) were pelleted (500g, 5 minutes, 4°C) and washed in 10mL of ice-cold 1x PBS (MP Biomedicals, U.S.A) before being resuspended in lysis buffer (1 mL containing; 10mM Tris (pH 7.4), 10mM NaCl, 3mM $MgCl_2$, 0.1mM EDTA (pH 8.0), 0.5% Igepal) and incubated on ice for 5 minutes. Nuclei were pelleted by centrifugation (500g, 3 minutes, 4°C) and the supernatant (cytoplasmic extract) was transferred to a fresh tube. Nuclei were washed once in Igepal free buffer (1ml containing; 10mM Tris (pH 7.4), 10mM NaCl, 3mM $MgCl_2$, 0.1mM EDTA (pH 8.0)) before being resuspended in nuclei lysis buffer (25µL containing; 400mM NaCl, 7.5mM $MgCl_2$, 0.2mM EDTA (pH 8.0), 1mM DTT) with added Complete EDTA-Free Protease Inhibitor Cocktail (Roche Applied Science, Switzerland). Samples were incubated on ice for 15 minutes with regular agitation. Cell debris was pelleted at 15,000g and the supernatant (nuclear extract) was transferred to a fresh tube.

Protein extracts were quantified by Bradford assay (Bradford, 1976). Briefly, extracts (2µl) were diluted 1:5 with MilliQ® water and combined with 1mL of diluted (1:5) Bio-Rad Protein Assay dye reagent concentrate (Bio-Rad Laboratories, U.S.A), absorbance was determined at 595nM by spectrophotometery (SpectraMax® Plus^{384}, Molecular Devices, U.S.A). Protein concentration was then determined relative to a

standard curve generated using a series of dilutions of Bovine Serum Albium (BSA, New England Biolabs, U.S.A) ranging in concentration from 0.00µg/mL to 2.00µg/mL.

2.3.2 SDS-PAGE and western blotting

Protein extracts (10-20µg) were prepared with 4x sample loading buffer (5µL containing; 240mM Tris HCl (pH 6.8), 40% glycerol, 8% SDS, 0.08% Bromophenol blue, 10% β-mercaptoethanol) to a total volume of 20µL and heated at 95°C for 5 minutes. Samples (20µL) were loaded on a 12% Mini-PROTEAN® TGX ™ pre-cast gel (Bio-Rad Laboratories, U.S.A) alongside 5 and 10µL of SpectraTM Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific, U.S.A) with sufficient SDS-PAGE running buffer (192mM glycine, 25mM Tris (pH 8.0) and 0.1% sodium dodecyl sulphate). Samples were initially electrophoresed for 15 minutes at 100 V followed by 150 V until completion.

Proteins were transferred from the gel to 0.45µM nitrocellulose membrane (Bio-Rad Laboratories, U.S.A) utilising a wet transfer. The transfer assembly consisted of the gel and membrane in direct contact surrounded by two layers of filter paper (Whatman, U.S.A) and a support pad on either side pressed together by the support grid. The apparatus was immersed in western transfer buffer (150mM glycine, 20mM Tris (pH 8.3) and 20% methanol) and transfer facilitated by 100 V for 90 minutes at 4°C.

The membrane was placed in blocking reagent (Blocking reagent (Roche Applied Science, Switzerland) diluted 1:10 in 1x TNT (10mM Tris (pH 8.0), 150mM NaCl, 0.05% Tween). and incubated overnight at 4°C with shaking. The membrane was then incubated with primary antibody (refer to table 3 for dilutions of specific antibody) followed by 3x 10 minute washes with 1x TNT at room temperature. The incubation and wash process was subsequently repeated for the appropriate secondary antibody (refer to table 3 for details). The membrane was then incubated for 5 minutes with SuperSignal® West Pico Stable Peroxide solution (2mL, Thermo Fisher Scientific,

U.S.A) and Pico Luminol/Enhancer solution (2mL, Thermo Fisher Scientific, U.S.A) with shaking. Excess chemiluminescent reagent was removed and the membrane was exposed for adequate lengths of time, up to 15 minutes using the Chemi-Smart 5000 (Vilber Lourmat, France).

2.3.3 Stripping and re-probing membranes for western analysis

For detection of additional proteins, membranes were washed for 10 minutes with 1X TNT then incubated with Restore^{TM} PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific, U.S.A) with vigorous shaking for 15 minutes to facilitate the removal of antibodies attached to the membrane. The membrane was then washed twice more with 1X TNT (10 minutes) before being placed in 1x Blocking Reagent (Roche Applied Science, Switzerland) and additional probing proceeded as described above (section 3.3).

2.4 Chromatin analysis

Table 2.4: Primers designed to promoter regions used for chromatin analysis

Name	Sequence (5' to 3')	Fragment Size (bp)
mGMA (Holloway et al. 2003)	**F:** GCCTGACAACCTGGGGGAAG **R:** TGATTAATGGTGACCACAGAACTC	**116**
mIL23a_pro (Ramirez-Carrozzi et al. 2009)	**F:** GCCTCTAGCCACAACAACCTC **R:** ATTCCCCTCCCTACATCATCTC	**95**
mIL6_pro		**93**

(Ramirez-Carrozzi et al. 2009)	**F:** AATGTGGGATTTTCCCATGA **R:** GCTCCAGAGCAGAATGAGCTA	
mIL10_pro (Ramirez-Carrozzi et al. 2009)	**F:** GCAGAAGTTCATTCCGACCA **R:** GGCTCCTCCTCCCTCTTCTA	**119**
RhoD (Poke et al. 2012)	**F:** ATATCTCGCGGATGCTGAAT **R:** GACAGACCAAGGCTGCTT	**136**

2.4.1 Chromatin accessibility by real time PCR (CHART-PCR)

Chromatin accessibility (CHART-PCR) assays were performed as previously described (Rao, Procko & Shannon 2001). Briefly, cells (up to $1x10^7$ per sample) were pelleted (500g, 5 minutes, 4°C) and washed in 10mL of chilled 1X PBS (MP Biomedicals, U.S.A) before being resuspended in chilled nuclei buffer (1 mL containing; 10mM Tris (pH 7.5), 10mM NaCl, 3mM $MgCl_2$, 0.1mM EDTA (pH 8.0), 0.5% Igepal, 0.15mM spermine and 0.5mM spermidine) and incubated on ice for 5 minutes. Nuclei were pelleted by centrifugation (500g, 3 minutes, 4°C) and washed once in MNase1 buffer (1mL containing; 10mM Tris (pH 7.5), 15mM NaCl, 60mM KCl, 0.15mM spermine and 0.5mM spermidine) before being resuspended in 200µl MNase1 buffer. An aliquot (94µL) of each sample was used for subsequent MNase1 digestion whilst an equal volume of each sample was used as an undigested control. Calcium chloride (1mM) was added to all samples.

For MNase1 digestion, samples were incubated with 1 unit of MNase1 (Roche Applied Science, Switzerland) in MNase1 buffer (5µL) for exactly 5 minutes before the reaction was quenched with the addition of stop buffer (20µL containing; 0.1M EDTA (pH 8) and 0.05M EGTA (pH 8)). MNase1 buffer alone (5µL) was added to undigested controls. All samples were then diluted to 200µL with MNase1 buffer and subjected to DNA extraction using the QIAamp DNA Blood Mini Kit (Qiagen, Netherlands)

according to manufacturer's directions. Genomic DNA (50ng per sample) was used for qPCR analysis (as per 2.2.3.1) with appropriate promoter primers (refer to table 2.4) and accessibility of DNA to MNase1 digestion was determined relative to undigested samples.

2.4.2 Chromatin Immunoprecipitation (ChIP)

ChIP analysis was performed as previously described (Chen et al. 2005). Briefly, proteins were cross-linked to DNA through treatment of EL4 T cells (15mL at a density of 5x 10^5 cells/mL) or RAW 264.7 cells (2x 75cm^2 flasks each with 20mL of media covering adherent cells at 80% confluence) with 37% formaldehyde (final concentration of 1%) for 10 minutes. Cross-linking was quenched through the addition of glycine (final concentration of 0.125M) and incubated at room temperature for 10 minutes. Adherent RAW 264.7 cells were recovered by scraping from the bottom of the flask. Cells were pelleted by centrifugation (500g for 5 minutes) and washed twice in ice cold PBS (MP Biomedicals, U.S.A) with Complete EDTA-Free Protease Inhibitor Cocktail (Roche Applied Science, Switzerland). Cells ($2.0x10^6$) were then re-suspended in 250µL sodium dodecyl sulphate (SDS) lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl. pH 8.1) with added Complete EDTA-Free Protease Inhibitor Cocktail (Roche Molecular Biochemicals, U.S.A) before being subjected to sonication using the settings on the Diagenode Bioruptor® (Diagenode, Belgium) empirically determined to generate DNA fragments 200 to 1000bp in length. ChIP dilution buffer (1mL containing; 0.01% SDS, 1.2mM EDTA (pH 8.0), 16.7mM Tris-HCl (pH 8.1), 1% Triton X-100, 167mM NaCl) and salmon sperm DNA/ protein A agarose slurry (60µL; Millipore Corporation, Germany) were added and incubated at 4°C for 1 hour on a rotating wheel in order to pre-clear samples. A total input sample was taken from the supernatant (100µL) and stored at -80ºC for use as a PCR control. Aliquots (300µL) of each sample were used for a no antibody control and each antibody treatment. Chromatin/bead/antibody mix was incubated overnight with rotation (4°C). For details of antibodies and concentrations used refer to table 2.5.

Immune complexes were recovered by incubation with salmon sperm DNA/ protein A agarose slurry (60µL; Millipore Corporation, Germany) for 2 hours and washed in a series of buffers; Low Salt Buffer (1mL containing, 0.1% SDS, 2.0mM EDTA, 20mM Tris- HCl (pH 8.1), 1% Triton X-100, 150mM NaCl), High Salt Buffer (1mL containing, 0.1% SDS, 2.0mM EDTA (pH 8.0), 20mM Tris-HCl (pH 8.1), 1% Triton X-100, 500mM NaCl), LiCl Buffer (1mL containing, 1.0mM EDTA pH 8.0, 10mM Tris-HCl (pH 8.1), 250mM LiCl, 1% Igepal, 1% Deoxycholate) then twice in TE Buffer (1mL containing, 1.0mM EDTA pH 8.0, 10mM Tris-HCl pH 8.1). All wash buffers were supplemented with Complete EDTA-Free Protease Inhibitor Cocktail (Roche Applied Science, Switzerland). Following the final TE wash, immune complexes were eluted in Elution Buffer (400µL, 1% SDS, 10mM $NaHCO_3$). Proteinase K (1µL, 20mg/mL; Qiagen, Netherlands) and NaCl (0.2M) were added to all samples and incubated overnight (65°C) to reverse cross-linking.

Immunoprecipitated DNA was purified by phenol/chloroform extraction using phase-lock gel light 1.5mL tubes (5 Prime, Germany) and ethanol precipitated overnight at -20°C before being washed in 70% ethanol, air-dried and resuspended in 50µL MilliQ® water (Millipore Corporation, Germany). DNA (5µL) was amplified by qPCR (as per section 2.2.3.1). Data was normalised to total input samples and analysed relative to no antibody controls and the silent Rhodopsin gene (refer to table 2.4 for primer sequences).

Table 2.5: Antibodies used for chromatin immunoprecipitation

Affinity	Host	Type	Amount per IP	Supplier (cat. No)
Anti - NFκB p65 (C-20)	Goat	Polyclonal	2µg	Santa Cruz Biotechnology, U.S.A (sc-272X)

Anti - c-Rel (C)	Rabbit	Polyclonal	2µg	Santa Cruz Biotechnology, U.S.A (sc-71X)
Anti – Histone H3	Rabbit	Polyclonal	1.8µg	Abcam, UK (ab1791)
Anti – acetyl-H3	Rabbit	Polyclonal	4µg	Millipore, Germany (06599)

2.5 Cloning of promoter regions

Upstream regions of the IL-6, IL-10 and IL-23A genes were cloned into the pXPG luciferase reporter plasmid (Bert et al. 2000). Primers were designed to specifically amplify the region approximately 1000bp upstream of the transcriptional start site of each gene using the National Centre for Biotechnology (NCBI) BLAST and Primer BLAST programs (http://blast.ncbi.nlm.nih.gov/). The New England BioLabs® (NEB) cutter V2.0 program (http://tools.neb.com/NEBcutter2/) was used to identify restriction enzymes which did not digest DNA within the promoter sequences. *Xho*1 and *Hind*III restriction enzymes were then selected and their recognition sequences added included at the 5' end of the primer sequences as indicated by underline in Table 2.6. Random nucleotides were then added on to the 5' end of the restriction enzyme sites to enhance digest efficiency.

Table 2.6: Promoter cloning primers

Name	Sequence (5' to 3')	Fragment Size (bp)
IL-6	**F:** TGCTACTCGAGGAGCTTCAAACACAAG **R:** TGCTACAAGCTTGCGGTTTCTGGAATTG	**1062 (+23)**

IL-10	**F:** TGCTACTCGAGCATTCCCTGGTCAACA **R:** TGCTACAAGCTTTAGTGCAAGAGCAAGT	**1042 (+23)**
IL-23A	**F:** TGCTACTCGAGGACGTCCAGATGGTGC **R:** TGCTACAAGCTTTAACTCTGTAGTCCGA	**1046 (+23)**

2.5.1.1 PCR amplification of promoter regions

Genomic DNA (50ng) was isolated from EL4 T cells using the QIAamp DNA Blood Mini Kit (Qiagen, Netherlands) and used as a template for the PCR amplification of the promoter regions. A 25µL Go-Taq® Green (12.5µL. Promega Corporation, U.S.A) reaction was employed with 0.2µM primers (Table 2.5). DNA was amplified using a Veriti® 96 Well Thermal Cycler (Applied Biosystems™, U.S.A) under the following cycling conditions: 1X 95ºC for 3 minutes, 35X 95ºC for 15 seconds followed by 56ºC 30 seconds and 72ºC for 1 minute. Following the last cycle samples were held at 72ºC for 10 minutes.

Annealing temperature was ascertained by gradient PCR for each primer pair. An annealing temperature of 56ºC was used for the IL-6 cloning primers whilst a temperature of 54ºC was selected for the IL-10 and IL-23A promoter cloning primers.

PCR product specificity was confirmed by electrophoresis through a 1% agarose gel in 1x TAE and PCR products were visualised using SYBR® safe DNA gel stain (Invitrogen™, U.S.A). Molecular weight standards *Hind*III λ (New England Biolabs, U.S.A) and HyperLadder II (Bioline, Australia) were electrophoresed alongside the PCR product for size comparison. Products were purified using the GFX™ DNA and

Gel Band Purification kit (GE Healthcare, UK) according to the manufacturer's directions.

2.5.1.2 Ligation

PCR products were ligated into the pXPG plasmid vector (Bert et al. 2000) which was first prepared using the Plasmid Maxi Kit (Qiagen, Netherlands). Both PCR products and vector were digested overnight with the *Xho*1 and *Hind*III restriction enzymes, followed by purification with the GFX^{TM} DNA and Gel Band Purification kit (GE Healthcare, UK). Ligations were performed using 50ng of vector and a 3:1 insert: vector ratio using 1µL of both T4 DNA Ligase and T4 Ligase buffer (New England BioLabs, U.S.A) in a 10µL reaction. Ligations were incubated overnight at room temperature.

2.5.1.3 Plasmid transformation

One Shot® TOP 10 Chemically Competent *E. coli* cells (Invitrogen^{TM}, U.S.A) were transformed with the ligated DNA according to the manufacturers' instructions. The *E. coli* cells (25µL) were thawed on ice. Ligated DNA (5µL) was added and incubated at on ice for 30 minutes. The cells were then heat-shocked at 42ºC for 30 seconds, incubated on ice for 2 minutes and 250µL of pre-warmed SOC medium (Invitrogen^{TM}, U.S.A) was added. The cells were incubated with shaking (37ºC, 1 hour) before being pelleted by centrifugation (15,000g, 5 minutes). Excess supernatant (170µL) was removed from cell pellets and the pellet was resuspended in residual SOC medium. Cells were then plated out on agar plates containing 50µg/mL ampicillin. Plates were incubated overnight at 37ºC to enable cell growth and subsequently stored at 4ºC.

2.5.1.4 Identification of recombinant plasmids

To select colonies which were successfully transformed with the recombinant plasmids colonies were screened utilising PCR, restriction enzyme digest and DNA sequencing.

2.5.1.5 PCR screening and restriction enzyme digest

Individual colonies were picked from each plate and inoculated into 10µL of MilliQ® (Millipore, Germany). Colonies were re-streaked onto agar plates containing 50µg/mL ampicillin and allowed to grow overnight at 37ºC before being stored at 4ºC for further use. The inoculated water was used as a template for PCR reaction as described in section 2.5.1.1. The PCR products were analysed by agarose gel electrophoresis and colonies that contained the recombinant plasmids were used for DNA isolation using the Wizard® Plus SV Minipreps DNA Purification System (Promega Corporation, U.S.A) and further analysis. A restriction enzyme digest was performed with the *Xho1* and *HindIII* restriction enzymes (New England BioLabs, U.S.A) on isolated DNA to confirm the plasmid identity. Digests were subjected to electrophoresis through a 1% agarose gel to confirm fragment sizes.

2.5.1.6 DNA sequencing

To confirm the successful generation of the recombinant plasmids identified by PCR screening and restriction enzyme digest (section 5.1.5.2) plasmid DNA (100ng) was sequenced to confirm the insert sequence. Forward and reverse primers were designed for the pXPG plasmid DNA so as to flank the promoter insert site. The sequencing reaction was carried out with either the forward primer (0.528µM) sequence: 5' TCATGTCTGGATCAGCCATATC 3' or the reverse primer (0.528µM) sequence: 5' TGGAAGACGCCAAAAACATAAAG 3'. The plasmid DNA was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, U.S.A) in a Veriti® 96 Well Thermal Cycler (Applied Biosystems™, U.S.A) using the following

reaction conditions: 1X 96ºC for 1 minute, 25X 96ºC 10 seconds, 50ºC 5 seconds and 60ºC for 4 minutes.

Sequencing reactions were purified using the CleanSEQ® beads (Agencourt® Bioscience Corporation, U.S.A) and resuspended in 40µL of MilliQ water. Purified sequencing reaction (30µL) were sequenced on the ABI™ 310 Genetic Analyzer (Applied Biosystems™, U.S.A) and sequences were analysed using Sequencher 4.10.1 DNA sequencing software (Gene Codes Corporation, U.S.A) to confirm insert sequence.

2.6 Luciferase Reporter Assay

The luciferase reporter plasmids containing the promoter regions of the IL-6, IL-10 and IL-23A cytokine genes cloned into pXPG were analysed for promoter activity and responsiveness to the NF-κB transcription factors, c-Rel and Rel-A using a luciferase reporter assay.

2.6.1 Plasmid Preparation and Cell Transfection

Each of the pXPG promoter plasmids (IL-6, IL-10 and IL-23A) were co-transfected with the Rel-A cFlag pcDNA3 (Addgene plasmid 20012) and c-Rel cFlag pcDNA3 (Addgene plasmid 20013), both of which have been described previously (Sanjabi et al. 2005). A non-active control plasmid RcCMV (Invitrogen™, U.S.A) was used in the absence of the Rel constructs to ensure equal DNA. Transfection with the luciferase activity control plasmid pGL3 (Promega Corporation, U.S.A) in which luciferase activity is under the control of the SV40 promoter and enhancer was performed alongside the other transfections to monitor transfection efficiency.

All plasmid DNA was prepared using the Plasmid Max Kit (Qiagen, Netherlands) from *E.coli* cultures grown with shaking in L broth (100mL) with 50µg/mL ampicillin (37ºC, overnight). Purified plasmid DNA was resuspended in TE buffer (100µL, Invitrogen™, U.S.A) and quantified using the NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, U.S.A). Plasmid DNA used for transfections was stored at -20ºC at a concentration of 1µg/µL.

2.6.1.1 Transfection of EL4 T cells

The EL4 cell line was passaged to reach a density of $5x10^5$ cells/mL at the time of transfection. Cells were pelleted by centrifugation (500g, 5 minutes) and resuspended at a concentration of $1.5x10^7$ cells/mL in pre-warmed RPMI 1640 medium (Invitrogen™, U.S.A) supplemented with fetal bovine serum (20%; JRH Biosciences, Australia), penicillin (100U/mL) and streptomycin (100µg/mL, Sigma-Aldrich, U.S.A). Purified plasmid DNA (5µg per plasmid) was pipetted onto the wall of a 0.4mm electroporation cuvette (Bio-Rad Laboratories, U.S.A). Cells (300µL) were added to the plasmid DNA and allowed to mix for 5 minutes. Cells were electroporated at 270V with a capacitance of 950µFarad and a resistance of infinity using a Gene Pulser Xcell™ (Bio-Rad Laboratories, U.S.A). Cells were recovered in 1mL of RPMI 1640 medium (Invitrogen™, U.S.A) supplemented with fetal bovine serum (10%; JRH Biosciences, Australia), penicillin (100U/mL) and streptomycin (100µg/mL, Sigma-Aldrich, U.S.A). Following 10 minutes of recovery, cells were returned to the incubator (37ºC, 5% CO_2) in 10mL of medium and incubated for 24 hours. Each transfection was performed in duplicate and combined into one flask after transfection.

2.6.1.2 Transfection of RAW 264.7 cells

The RAW 264.7 cell line was passaged to reach 70% confluence at the time of transfection. RAW 264.7 cells were transfected using a protocol described previously

(Smale 2010b). Briefly, adherent cells were removed with a cell scraper and pelleted (500g, 5 minutes). Cells were resuspended in pre-warmed RPMI 1640 medium (Invitrogen^{TM}, U.S.A) supplemented with fetal bovine serum (20%; JRH Biosciences, Australia), penicillin (100U/mL) and streptomycin (100µg/mL, Sigma-Aldrich, U.S.A) at a concentration of $3.75x10^7$ cells/mL Purified plasmid DNA (5µg per plasmid) was pipetted onto the wall of a 0.4mm electroporation cuvette (Bio-Rad Laboratories, U.S.A). Cells (200µL) were added to the plasmid DNA and allowed to mix for 5 minutes. Cells were electroporated at 250V with a capacitance of 950µFarad and a resistance of infinity using a Gene Pulser Xcell^{TM} (Bio-Rad Laboratories, U.S.A). Following transfection cells were washed in 1X PBS (MP Biomedicals, U.S.A) and resuspended in RPMI 1640 medium (Invitrogen^{TM}, U.S.A) supplemented with fetal bovine serum (10%; JRH Biosciences, Australia), penicillin (100U/mL) and streptomycin (100µg/mL, Sigma-Aldrich, U.S.A) and returned to the incubator (37ºC, 5% CO_2) in 10mL of medium and incubated for 24 hours. Each transfection was performed in duplicate and combined into one flask after transfection.

2.6.2 Preparation of cell lysates for reporter assay

At 24 hours post-transfection cells were pelleted by centrifugation (500g, 5 minutes at 4°C) and washed twice with ice-cold PBS before re-suspension in 100µL of cell lysis buffer (1X Cell Culture Lysis Reagent, Promega Corporation, U.S.A). Cell lysates were then transferred to a 1.5mL microcentrifuge tube and kept on ice. Tubes were vortexed briefly to ensure complete cell lysis and centrifuged (12,000g for 2 minutes) at room temperature to facilitate separation of cell debris. Supernatant was transferred to a new 1.5mL microcentrifuge tube and stored at -80°C.

2.6.3 Luciferase activity assay

Protein concentration of lysates was determined by Bradford assay as outlined in section 3.2. Luciferase activity was analysed using the Luciferase Assay System

(Promega Corporation, U.S.A). Luciferase Assay Substrate was reconstituted with the Luciferase Assay Buffer as directed by the manufacturer. Cell lysate (30µg) was aliquoted into an opaque white, 96-well plate (Greiner Bio-one, Germany), in duplicate and the total volume made up to 100µL with 1x cell culture lysis buffer (Promega Corporation, U.S.A). Luciferase activity was measured following the addition of 100µL reconstituted Luciferase Assay Reagent (Promega Corporation, U.S.A) using a Turner Biosystems Veritas™ Microplate Luminometer (Turner Biosystems Incorporated, U.S.A).

2.7 Data Analysis

All basic data analysis was performed in Microsoft Excel 2010. Unless otherwise specified a paired Students T. Test was used to determine significance from non-stimulated. Unless otherwise specified, p values shown are for two tailed T. Tests. A p value of less than 0.05 was considered significant.

3 Divergent activation requirements for GM-CSF gene expression in T cells and macrophages

3.1 Introduction

The initiation, duration and resolution of an immune response is dependent on the coordinated induction of immune gene expression programs. A key control point in the induction of cytokine gene expression is at the level of gene transcription, through the assembly of precise transcription initiation complexes at gene regulatory regions (Carey 1998; Litterst et al. 2005; Ptashne & Gann 1997). However, the assembly of such transcription complexes must take place within the chromatin environment established at the gene. Thus gene responses are dependent on the interaction between transcription factors, transcription initiation complexes and the chromatin platform (Li, Carey & Workman 2007; Lim et al. 2013; Natoli 2009). The chromatin structure itself is further modified by interaction with histone modifying enzymes and chromatin remodelling complexes (Cockerill 2011; Gardner, Allis & Strahl 2011; Kouzarides 2007).

The inflammatory cytokine, Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) otherwise referred to as Colony Stimulating Factor 2 (CSF2) is produced at high levels in T cells following immune activation. As such, the *GM-CSF* gene has proved a valuable model with which to study how inducible gene expression is regulated both at the level of transcription (Himes et al. 1996; Shannon, Himes & Coles 1995) and by chromatin structure (Brettingham-Moore et al. 2005; Brettingham-Moore et al. 2008; Cakouros et al. 2001; Cockerill 2004; Holloway et al. 2003). Regulation of the rapid activation of *GM-CSF* gene expression has been attributed to a proximal promoter, encompassing 100bp upstream of the transcriptional start site, in addition to an upstream enhancer (Shannon et al. 1997). The activation of *GM-CSF* gene transcription

and the associated changes in chromatin architecture at both the promoter and enhancer regulatory regions of the gene, have been extensively studied in T cells. Increased gene transcription is induced via signalling through the T cell receptor and to co-stimulatory molecules which activate the protein kinase C (PKC) and calcium signalling pathways (Brettingham-Moore et al. 2005; Shannon et al. 1997). Whilst low basal levels of gene expression are permissible without chromatin remodelling, higher levels of transcription occur in association with changes in the chromatin architecture of the promoter and enhancer regions (Cockerill et al. 1993). Changes in nucleosome occupancy at the promoter region are detected by an increase in sensitivity of the promoter DNA to digestion by MNase1 (Holloway et al. 2003). This increased sensitivity is in part attributable to the loss of core histone proteins from the region indicating that increased gene transcription is enabled by chromatin remodelling events (Brettingham-Moore et al. 2008; Chen et al. 2005; Holloway et al. 2003; Poke et al. 2012). Prior to remodelling, the promoter is marked by high levels of histone acetylation which may be recognised by and recruit activating factors to the promoter region. The chromatin remodelling protein, BRG1, is a member of the SWI/SNF complex and contains an acetylation recognising bromodomain which associates with the promoter region (Brettingham-Moore et al. 2005; Brettingham-Moore et al. 2008).

As part of the SWI/SNF complex, BRG1 is able to utilise ATPase to disrupt DNA/nucleosome interaction via complete displacement, nucleosome sliding or unravelling, which may directly alter gene expression (Saha, Wittmeyer & Cairns 2006; Wurster, Precht & Pazin 2011). BRG1 is essential for embryonic development and is known to have an important regulatory role in T cell development (Bultman et al. 2000; Chi et al. 2002; Chi et al. 2003) as well as maintaining functionality of mature cells of the haematopoietic lineages (De et al. 2011; Ramirez-Carrozzi et al. 2006; Wurster & Pazin 2008). Whilst remodelling enzymes, such as BRG1, are thought to bind DNA non-specifically, they may be recruited to targeted regions through interaction with modified histones (such as histone acetylation), transcription factors or non-coding RNA (Hassan et al. 2001; Ho & Crabtree 2010; Lee et al. 2010; Singh et al. 2007; Wurster & Pazin 2008; Wysocka et al. 2006; Yudkovsky et al. 1999). BRG1 has been

identified as a functional regulator of the *GM-CSF* gene in T cells, where it is thought to mediate the changes in nucleosome occupancy of the promoter region, allowing the transcription machinery to assemble and facilitate gene transcription (Brettingham-Moore et al. 2008; Holloway et al. 2003).

The NF-κB transcription factors are key mediators of gene expression throughout the immune system and have a critical role in *GM-CSF* transcription (Cakouros et al. 2001; Ghosh, May & Kopp 1998; Holloway et al. 2003; Shannon, Himes & Coles 1995). c-Rel appears to be particularly crucial for *GM-CSF* transcription, as c-Rel depleted T cells are unable to increase *GM-CSF* expression in response to stimulation (Gerondakis et al. 1996). In addition, c-Rel has been shown to be required for chromatin remodelling events which precede increased *GM-CSF* transcription (Brettingham-Moore et al. 2005; Poke et al. 2012). Furthermore, an interaction between the Rel-A subunit and BRG1 has been observed at a distal enhancer region of the *GM-CSF* and *IL-3* locus which is also associated with increased gene expression (Wurster, Precht & Pazin 2011).

GM-CSF expression is not restricted to the T cell lineage, with expression also inducible in response to stimuli in several other cell types including macrophages, mast cells and fibroblasts (Cockerill 2004; Shannon et al. 1997). However the activation requirements in other cell types are less understood. Available data suggests that in macrophages, rather than being dependent on BRG1 chromatin remodelling, *GM-CSF* gene activation is SWI/SNF independent. Additionally, *GM-CSF* gene expression has been classified as independent of new protein synthesis and therefore a primary response gene in macrophages (Ramirez-Carrozzi et al. 2009). In contrast, in T cells *GM-CSF* is a secondary response gene with gene expression dependent on the synthesis of new proteins (Brettingham-Moore et al. 2005).

These studies suggest that *GM-CSF* gene activation is regulated by fundamentally different mechanisms in macrophages compared to T cells. Therefore the aim of this chapter is to characterise the activation requirements of the *GM-CSF* gene in macrophage cells in comparison to T cells.

3.2 Results

3.2.1 GM-CSF gene expression is induced in T cells and macrophages

To confirm that *GM-CSF* gene transcription is inducible in both T cells and macrophages and to establish cell models in which to investigate the mechanisms of *GM-CSF* gene regulation, gene responses were analysed by RT-qPCR using RNA extracted from both murine cell lines and primary cells prepared from male C57/BL6 mice. In the EL4 T cell line (Figure 3.1.A) an increase in *GM-CSF* mRNA levels is observed in response to 4 hours of treatment with PMA and calcium ionophore (PI), ($p<0.01$). Treatment with PI activates the protein kinase C and calcium signalling pathways respectively, which operate downstream of T cell receptor signalling (Takahama & Nakauchi 1996; Tsuboi et al. 1994). Similarly, CD4+ T cells prepared from mouse splenocytes produced increased *GM-CSF* mRNA levels in response to 4 hours of PI treatment (Figure 3.1.B, $p<0.05$). In contrast, in RAW 264.7 macrophages exposed to PI for 4 hours there was no discernible increase in *GM-CSF* transcript levels (Figure 3.2.A). The bacterial cell wall component, lipopolysaccharide (LPS) is routinely used to illicit immune responses in macrophage cells as it signals through Toll Like Receptor 4 (TLR4), and has previously been documented to induce *GM-CSF* gene expression (Li et al. 2012; Ramirez-Carrozzi et al. 2006). In keeping with this, RAW 264.7 macrophage cells stimulated for 4 hours with LPS displayed increased *GM-CSF* mRNA levels (Figure 3.2.B, $p= 0.050$). Primary macrophage cells were derived from C57/BL6 mouse bone marrow cells differentiated by culturing in M-CSF conditioned medium. Following 5 days of differentiation >90% of cells stained positive for the monocyte/macrophage marker CD11b (Figure 3.3.A). Treatment of these cells with LPS for 4 hours also produced a significant increase in *GM-CSF* mRNA levels (Figure 3.3.B. $p<0.05$). The relative levels of *GM-CSF* mRNA are greater in T cells when

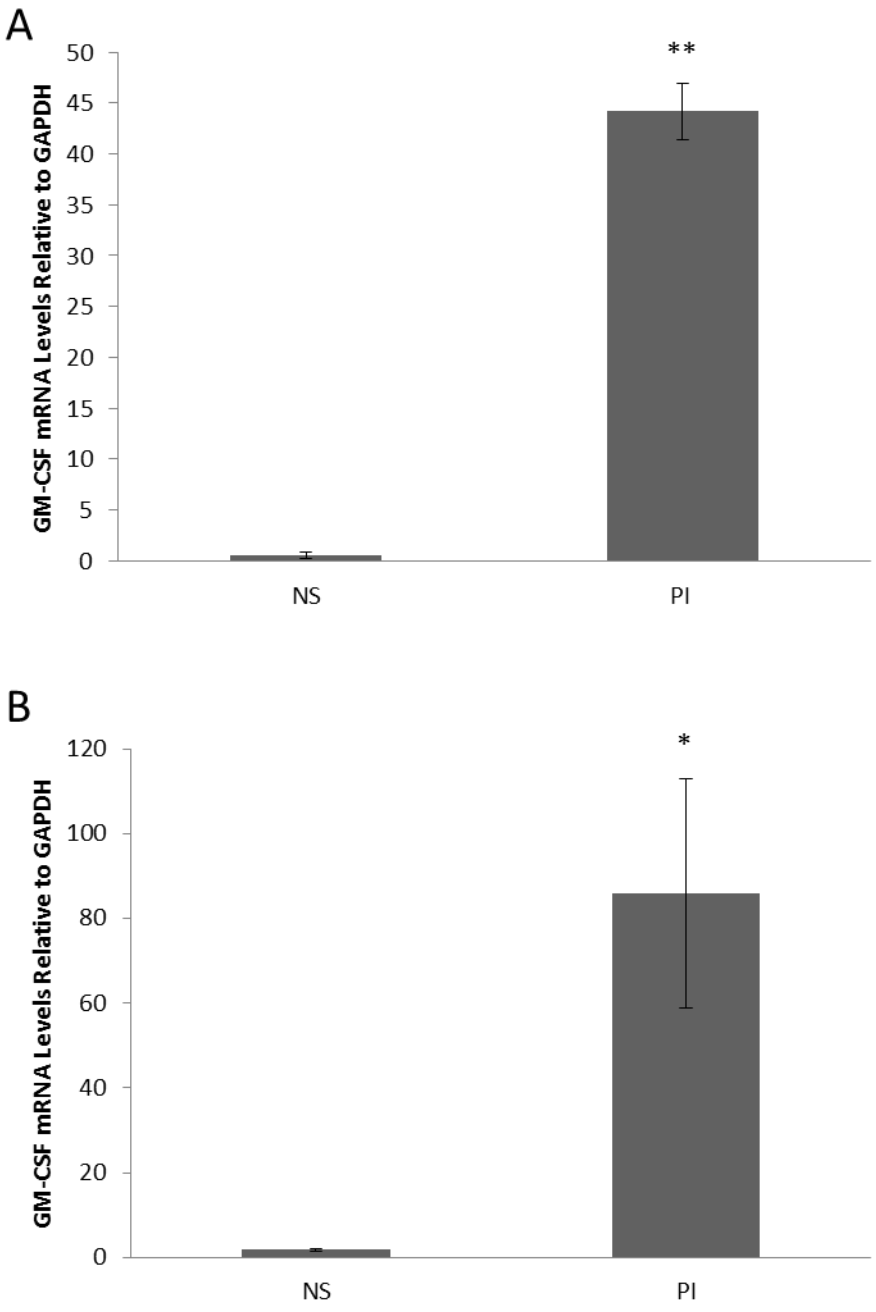

Figure 3.1: GM-CSF mRNA induction in T cells. (A) Murine EL4 T cells were either left non-stimulated (NS) or treated with PMA and calcium ionophore (PI) for 4 hours. (B) Primary CD4+ T cells were either left NS or treated with PI for 4 hours. In each case RNA was isolated and GM-CSF mRNA levels were determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown. Significance from NS was determined using a Students T. Test (single tailed). * $p<0.05$. ** $p<0.01$.

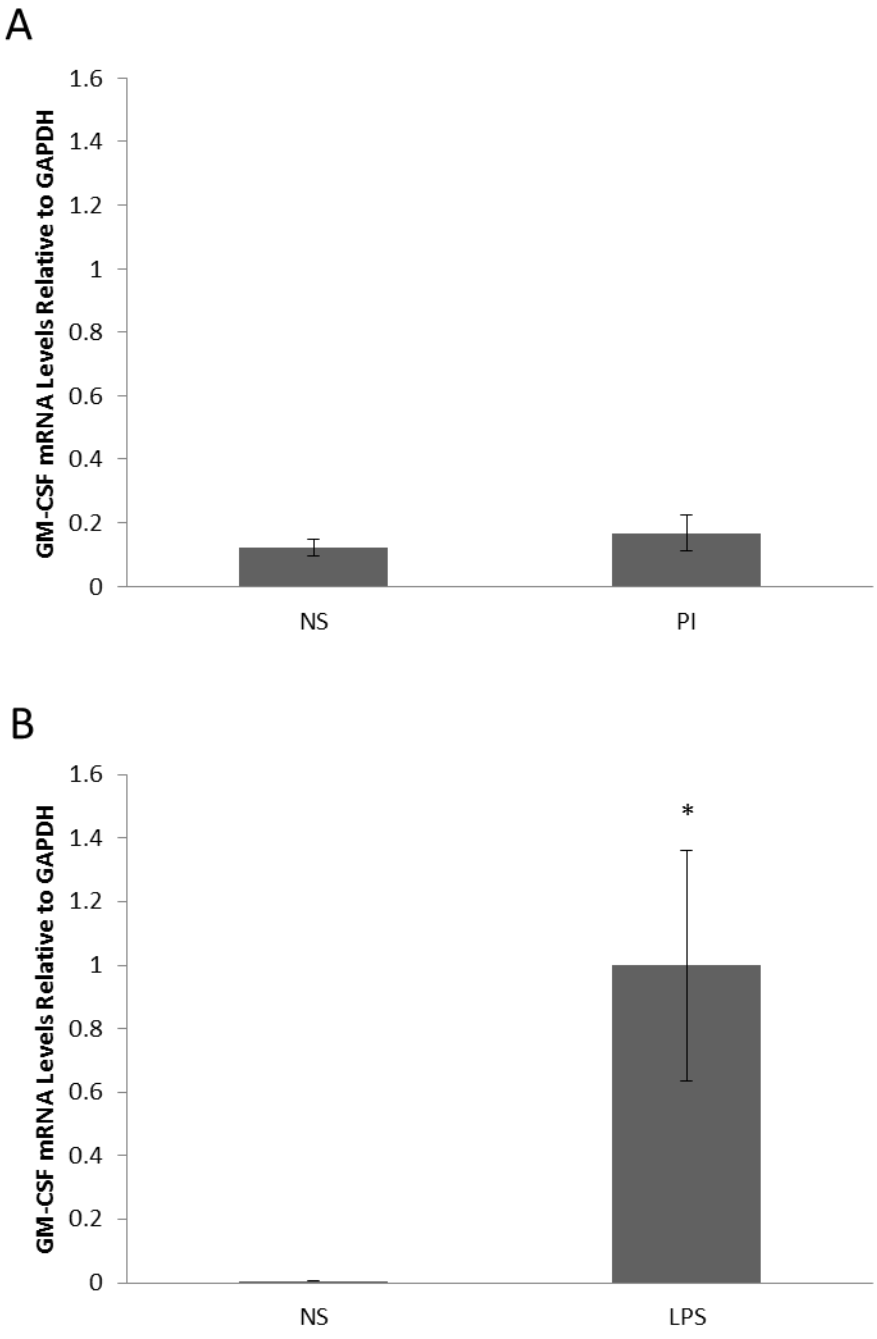

Figure 3.2: GM-CSF mRNA induction in macrophage cells. (A) RAW 264.7 cells were either left non-stimulated (NS) or treated with PMA and calcium ionophore (PI) for 4 hours. (B) RAW 264.7 cells were either left NS or treated with lipopolysaccharide (LPS) for 4 hours. In each case, RNA was extracted and GM-CSF mRNA levels were determined utilising RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown. Significance from NS was determined using a Students T. Test (single tailed). * $p<0.05$.

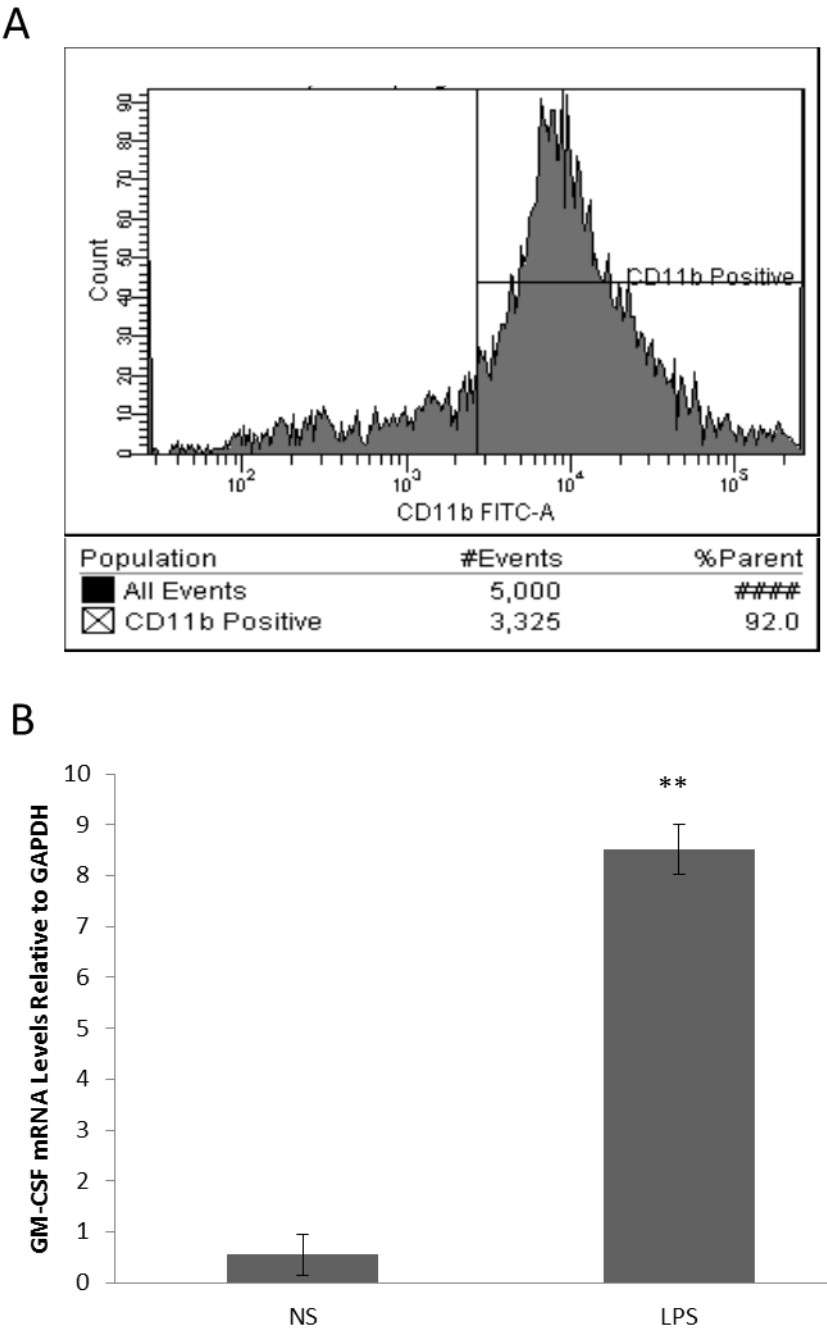

Figure 3.3: GM-CSF mRNA induction in bone marrow derived macrophages (BMDM). (A) Bone marrow cells were extracted from C57/BL6 mice and differentiated in M-CSF conditioned media. Differentiated cells were detected with antibodies to the monocyte/macrophage marker CD11b utilising flow cytometry. (B) BMDM cells were either left unstimulated (NS) or treated with LPS for 4 hours. In each case, RNA was isolated and GM-CSF mRNA levels were determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown. Significance from NS was determined using a Students T. Test (single tailed). ** $p<0.01$.

compared to macrophages. This is not attributable to differences in the house-keeping gene *GAPDH* to which expression was normalised. Rather this is a reflection of lower absolute *GM-CSF* mRNA levels induced in macrophage cells.

3.2.2 Kinetics of GM-CSF activation is similar in T cells and macrophage cells

The kinetics of *GM-CSF* gene activation were analysed in T cells and macrophages in order to determine if this is reflective of the switch between a secondary to a primary response gene as reported in the literature. Changes in *GM-CSF* mRNA levels were monitored over a 24 hour time course in both EL4 T cells and RAW 264.7 macrophage cells following stimulation. Cells were collected for RNA extraction both prior to stimulation (NS) as well over a 24 hour period post stimulation. Basal transcript levels were low in T cells (Figure 3.4.A) and not detectible in macrophage cells (Figure 3.4.B). In response to stimulation an increase in *GM-CSF* mRNA levels was observed in both cell types, however the amplitude of maximal expression varied between individual experiments as well as between cell types. Therefore in order to effectively compare the kinetics of the response between the two cell types, gene expression was normalised to expression at 4 hours, which was set to 100 in each case (unadjusted data is available in Appendix B, page 178). The 4 hour time point was selected as at this time, a significant increase in *GM-CSF* mRNA levels was observed in both cell types. As previously documented in T cells, *GM-CSF* mRNA levels increased in response to PI treatment and a peak in *GM-CSF* transcription was observed after 8 hours of PI treatment and had decreased by 24 hours (Brettingham-Moore et al. 2005; Brettingham-Moore et al. 2008), as depicted in Figure 3.4.A. A similar activation profile was observed in RAW 264.7 macrophage cells, with mRNA levels peaking at 8 hours and decreasing by 24 hours post stimulation (Figure 3.4.B). As no discernible difference in activation kinetics of *GM-CSF* was observed between the two cell types, subsequent experiments utilised the 4 hour time point for stimulation.

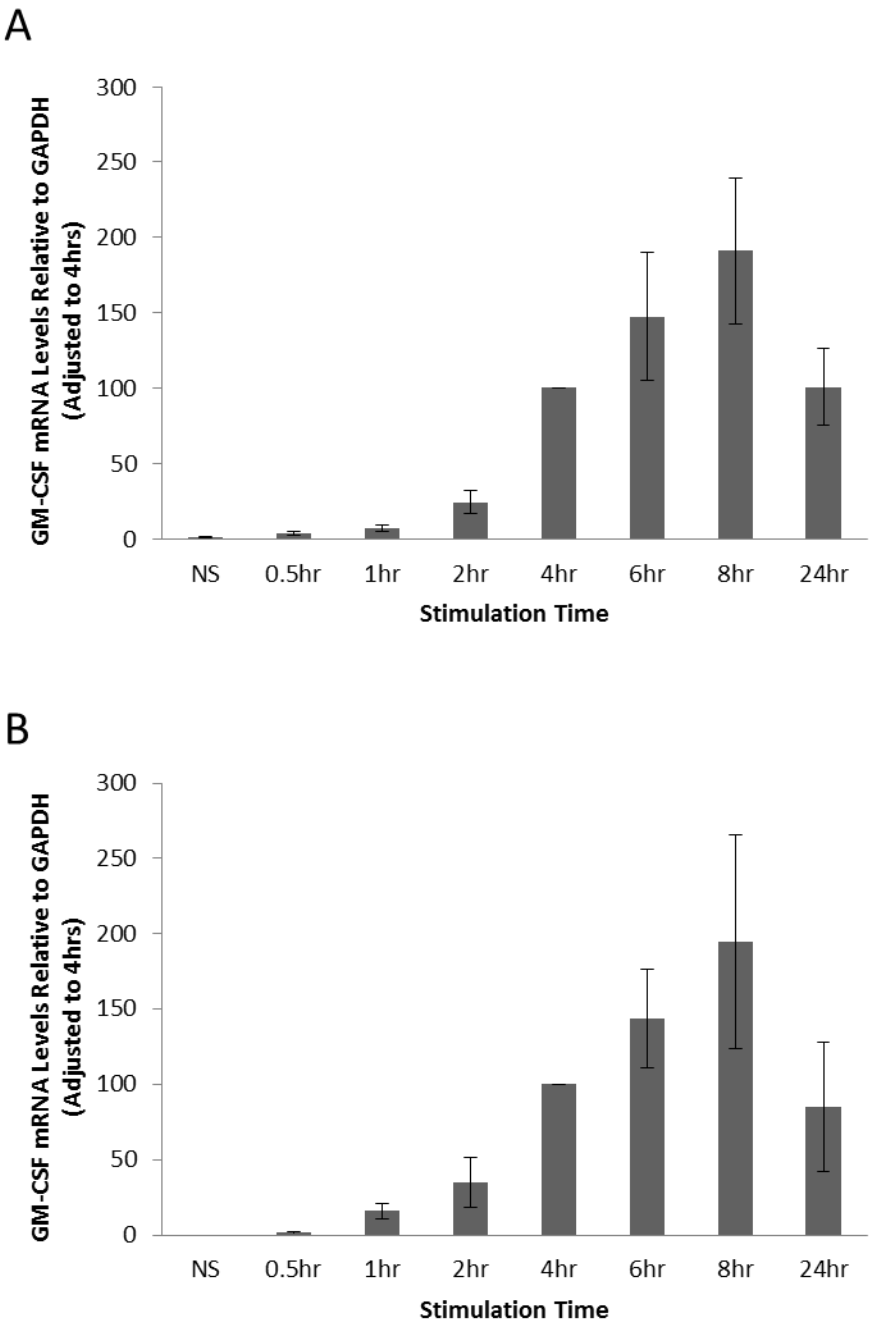

Figure 3.4: Activation kinetics of the GM-CSF gene in T cell and macrophage cell lines. (A) Murine EL4 T cells were incubated with PI and (B) RAW 264.7 macrophages were incubated with LPS. Cells were either left unstimulated (NS) or stimulated for the indicated times. In each case, RNA was isolated and GM-CSF mRNA levels determined by RT-qPCR and normalised to GAPDH. Gene expression is graphed relative to 4 hours of stimuli. Mean and SEM of at least 3 replicates is shown.

3.2.3 GM-CSF gene transcription and reliance on new protein synthesis

While no difference in activation kinetics was observed, previous work has suggested that *GM-CSF* is a secondary response gene in T cells (Brettingham-Moore et al. 2005), but a primary response gene in macrophages (Ramirez-Carrozzi et al. 2009). In order to confirm this divergence between the two cell types, gene expression responses were studied in EL4 T cells and RAW 264.7 macrophages. Gene transcription was examined in the presence of the protein synthesis inhibitor cycloheximide (CHX) to determine the reliance on the translation of new proteins for induction of *GM-CSF* gene expression. Cells were either left un-treated or pre-treated with CHX for 30 minutes. Following pre-treatment cells were either left unstimulated or stimulated with PI (EL4 T cells) or LPS (RAW 264.7 cells) for 4 hours.

In T cells, the increase in *GM-CSF* mRNA levels in response to stimulation was reduced in the presence of CHX (Figure 3.5.A; $p<0.05$). In contrast, CHX treatment of RAW 264.7 cells enhances the transcriptional response to LPS (Figure 3.5.B; $p= 0.212$), with enhanced transcription observed as soon as 1 hour post stimulation (data not shown). These results demonstrate that whilst *GM-CSF* mRNA levels are inducible in both T cells and macrophages, the requirements for *GM-CSF* gene transcription vary between the two cell types. The divergence in CHX dependence may be reflective of differences in proteins required for gene activation or differences in the basal levels of these proteins in the different cell types. To determine if this response is unique to the RAW 264.7 macrophage cell line, the activation response in the presence of CHX was also determined in primary bone marrow derived macrophages (BMDMs).

BMDM cells have a similar transcription profile to the RAW 264.7 macrophage cells with increased *GM-CSF* mRNA levels in the presence of LPS ($p<0.01$). CHX treatment alone increased *GM-CSF* mRNA levels, whilst superinduction of *GM-CSF* mRNA was observed with combined CHX and LPS treatment (Figure 3.6.A). These results confirm that the RAW 264.7 macrophage data is not limited to the immortalised cell line alone and can be replicated in primary cultured macrophages.

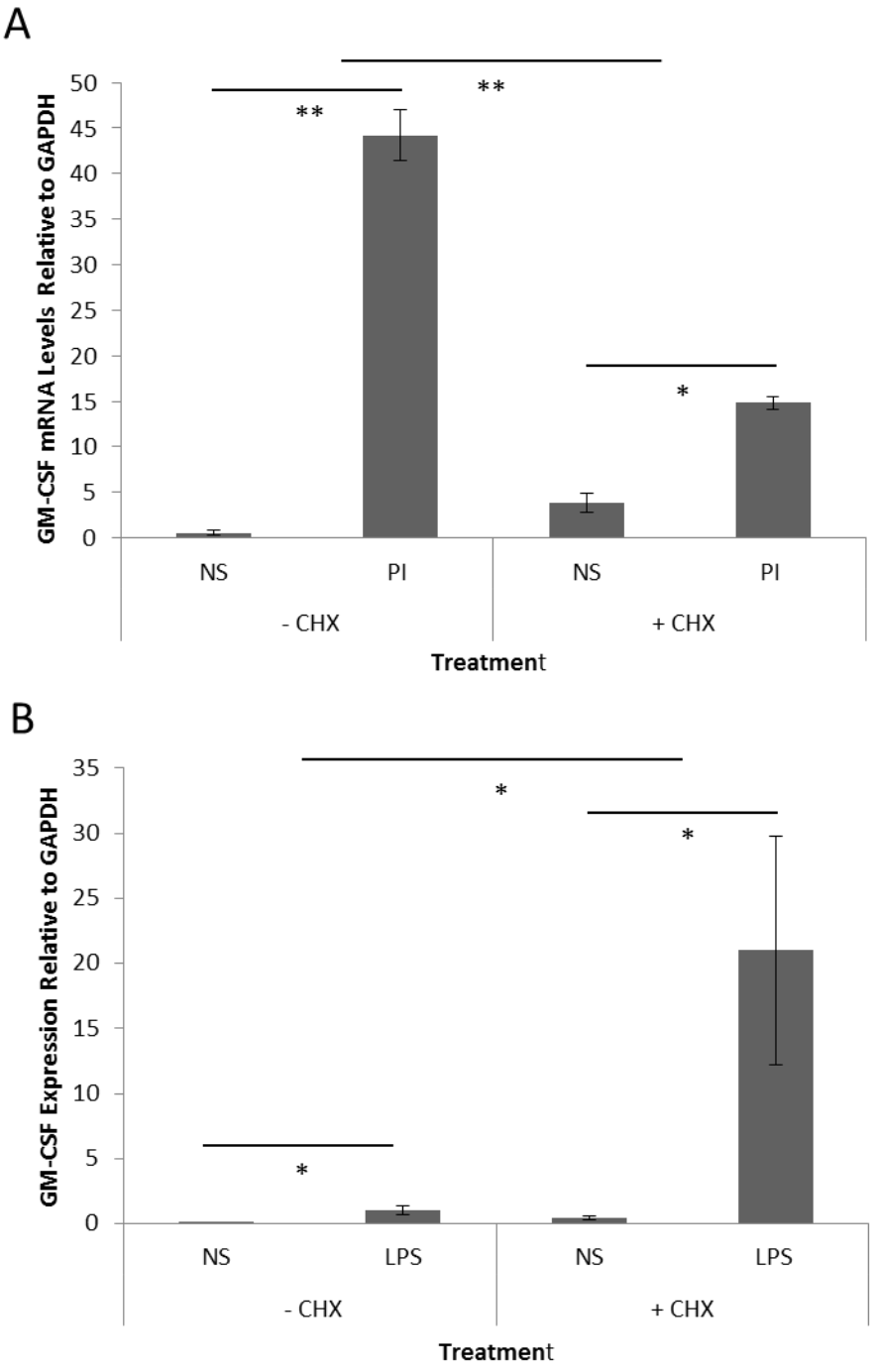

Figure 3.5: Cycloheximide (CHX) dependence of GM-CSF gene activation is different in macrophages compared to T cells. (A) Murine EL4 T cells were either left untreated or treated with CHX for 30 minutes before incubating with or without PI for 4 hours. (B) Murine RAW 264.7 macrophage cells were either left untreated or treated with CHX for 30 minutes before incubating with or without LPS for 4 hours. In each case, RNA was isolated and GM-CSF mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown. The change in relative mRNA levels from NS to stimulated is compared between –CHX and +CHX treatments Students T. Test. * $p < 0.05$. ** $p < 0.01$.

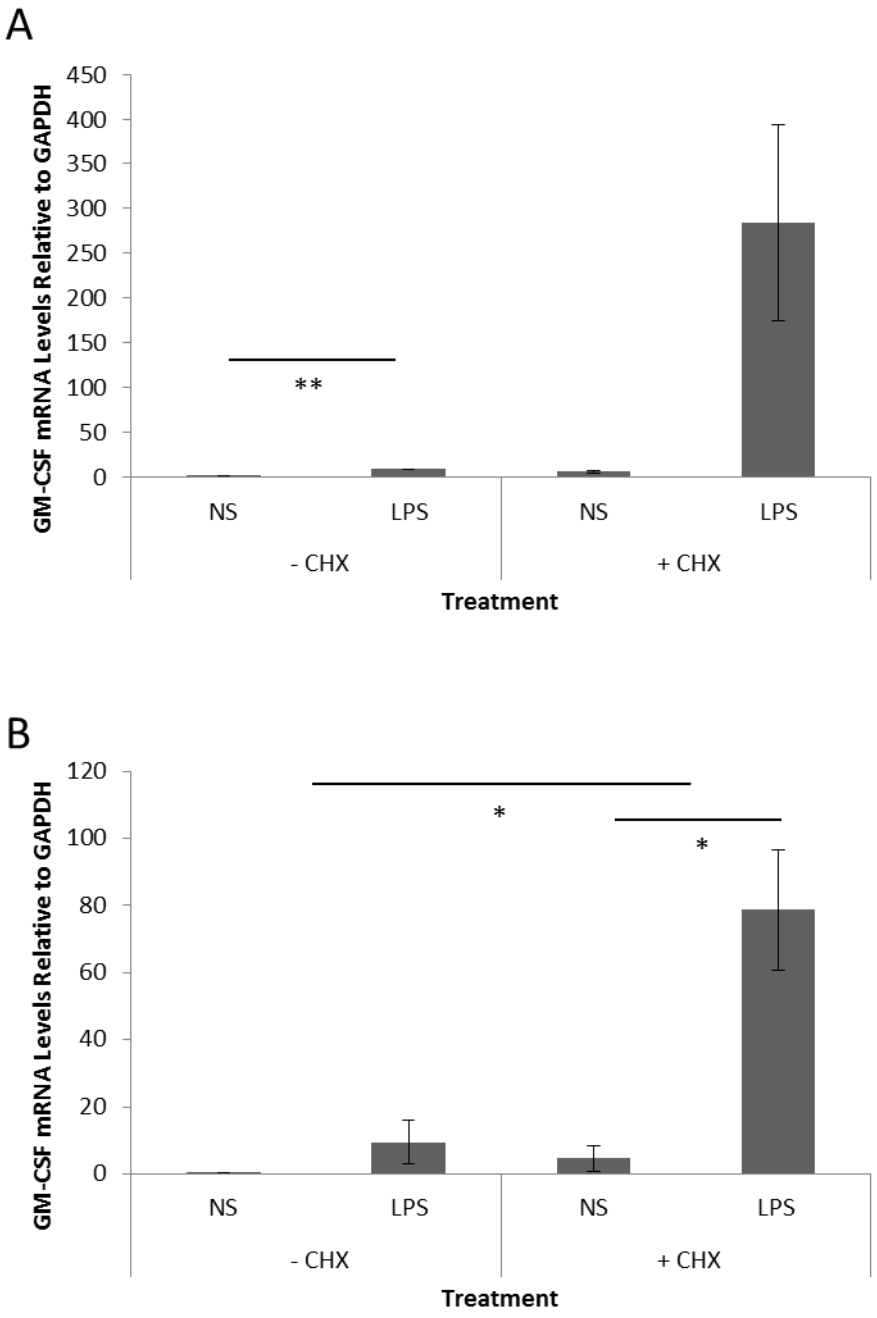

Figure 3.6: Cycloheximide (CHX) dependence of GM-CSF gene activation in primary cells. (A) Bone marrow derived macrophage cells and (B) Murine embryonic fibroblasts were cultured from C57/BL6 mice and were either left untreated or treated with CHX for 30 minutes before incubating with or without LPS for 4 hours. In each case, RNA was isolated and GM-CSF mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown. The significance of the change in gene expression upon stimulation is compared between –CHX and +CHX treatments by Students T. Test * $p<0.05$. ** $p<0.01$.

Fibroblast cells are also documented as producing *GM-CSF* in response to immune stimuli (Shannon et al. 1997). In order to confirm this, murine embryonic fibroblasts (MEFs) were extracted from day 14 mouse embryos. Cultured MEF cells were treated with CHX and LPS as described for the BMDM. An increase in *GM-CSF* mRNA levels in response to LPS stimuli was observed in MEFs (Figure 3.6.B. $p= 0.175$). In the presence of CHX the *GM-CSF* mRNA levels were significantly enhanced ($p<0.05$) indicating that *GM-CSF* superinduction in the presence of CHX is not limited to macrophage cells.

Therefore, in T cells *GM-CSF* is classified as a secondary response gene, reliant on de novo protein synthesis prior to increased gene transcription. In contrast, in macrophage and fibroblast cells *GM-CSF* is a primary response gene with increased expression in response to stimulation independent of new protein synthesis.

3.2.4 NF-κB nuclear localisation differs in T cells compared to macrophages

The c-Rel transcription factor is a key member of the NF-κB family and has been recognised as being of particular importance in BRG1 chromatin remodelling events observed at the *GM-CSF* promoter in T cells (Brettingham-Moore et al. 2005; Poke et al. 2012). As *GM-CSF* gene expression is thought to be SWI/SNF independent in macrophages (Ramirez-Carrozzi et al. 2009), different availability/requirement for c-Rel may be associated with the switch from being reliant on new protein synthesis in T cells to being independent of protein synthesis in macrophages. Prior to activation the NF-κB family transcription factors are tethered in the nucleus by the inhibitory IκB proteins. Upon activation, a series of phosphorylation and ubiquitination events occur leading to the degradation of IκB proteins and unmasking of the NF-κB nuclear translocation sequence. NF-κB transcription factors are then shuttled into the nucleus

where interactions with DNA and transcription machinery may take place (Karin & Ben-Neriah 2000).

To determine whether differences in NF-κB activation in the two cell types explains the differences in protein synthesis dependence, western blot analysis was performed on cytoplasmic and nuclear protein extracts prepared from both untreated and CHX treated EL4 T cells and RAW 264.7 macrophages both non-stimulated (NS) and stimulated (PI and LPS). Blots were probed with antibodies against the c-Rel and Rel-A family members as well as the constitutively nuclear transcription factor, SP1 and β-actin as loading controls (Figure 3.7).

In EL4 T cells, Rel-A was detectible in the cytoplasm and at low levels in the nucleus prior to stimulation (Figure 3.7.A). PI treatment increased the amount of Rel-A detected in both the nuclear fraction and the cytoplasmic fraction (Figure 3.7.A). In the presence of CHX, PI treatment results in an increase in nuclear Rel-A, indicating translocation of Rel-A to the nucleus is not reliant on the synthesis of new proteins. However, no increase in Rel-A is discernible in the cytoplasmic extract suggesting that the increase in cytoplasmic Rel-A in response to PI stimulation is the product of new Rel-A synthesis. In comparison, c-Rel is detectible at low levels in the EL4 T cell cytoplasmic fraction in the non-stimulated samples but undetectable in the nucleus (Figure 3.7.A). An increase in nuclear c-Rel is detected upon PI treatment however this response is inhibited in EL4 T cells pre-treated with CHX (Figure 3.7.A). These data suggested that in EL4 T cells the c-Rel transcription factor requires protein synthesis for nuclear activation as only very low levels are present in the cytoplasm prior to stimulation. It would therefore be expected that genes over which c-Rel exhibits regulatory control, such as *GM-CSF*, will be affected by CHX treatment in T cells.

The house-keeping protein, β-actin was used as a loading control for all samples whilst transcription factor Sp1, which is constitutively present in the nucleus, was used as a nuclear loading control for the western analysis. In EL4 T cell extracts β-actin is detectible in all nuclear and cytoplasmic fractions although at slightly lower levels in

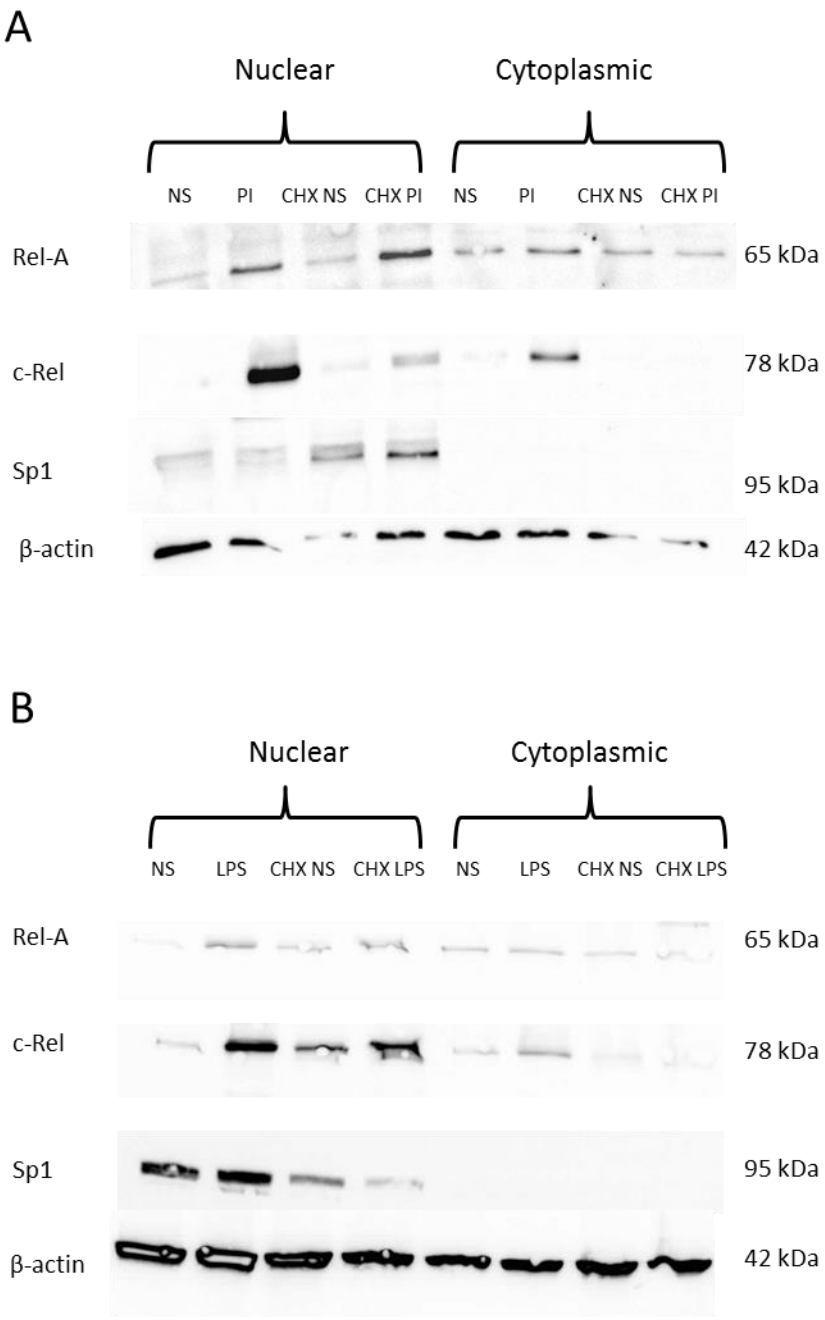

Figure 3.7: Nuclear localisation of NF-κB transcription factors. (A) EL4 T cells were either left untreated (NS) or treated with CHX for 30 minutes before incubating with or without PI for 4 hours. (B) RAW 264.7 macrophage cells were either left untreated or treated with CHX for 30 minutes before incubating with or without LPS for 4 hours. Nuclear and cytoplasmic protein extracts were prepared and western blot analysis was performed with antibodies against proteins of interest, Rel-A, c-Rel, Sp1 and β-actin, as indicated.

the CHX treated samples indicating that the synthesis of β-actin is inhibited by CHX. The nuclear loading control, Sp1, was absent from the cytoplasmic fractions indicating no nuclear contamination.

In the extracts prepared from RAW 264.7 cells, Rel-A is detectible in both the nuclear and cytoplasmic fractions in all treatments (Figure 3.7.B). An increase in Rel-A in response to LPS stimulation is observed, however not to the level seen in T cells. In contrast to what was seen in T cells, nuclear localisation does not appear to be reliant on the synthesis of new proteins (Figure 3.7.B). In RAW 264.7 macrophage cells an increase in nuclear c-Rel occurs in response to 4 hours of LPS treatment and an increase in cytoplasmic c-Rel is also observed (Figure 3.7.B). Nuclear localisation is still observed in the presence of CHX (Figure 3.7.B). Further, in the presence of CHX alone an increase in nuclear c-Rel is observed suggesting that in RAW 264.7 cells, c-Rel translocation is permitted in the absence of new protein synthesis.

In the RAW 264.7 extracts, β-actin is detectible at comparable levels between all nuclear and cytoplasmic fractions suggesting that an equal quantity of protein was loaded per well. The nuclear loading control, Sp1, is more variable. In contrast to what was observed in the T cell fractions, a greater amount of Sp1 is detected in the RAW 264.7 nuclear fractions which were not pre-treated with CHX (both NS and LPS treated). If CHX treated nuclear samples do not contain less protein then this further supports the observation that CHX enhances the nuclear localisation of c-Rel in macrophages but inhibits c-Rel translocation in T cells.

These results support the hypothesis that the differences in protein synthesis requirements of the *GM-CSF* gene may be partially explained by the different effect of CHX treatment on c-Rel localisation in T cells compared to macrophages. In T cells CHX treatment inhibited both the synthesis of new cytoplasmic c-Rel and the nuclear localisation of c-Rel in response to PI stimulation. In macrophage cells, nuclear

localisation of c-Rel in response to LPS stimulation was not perturbed by CHX treatment.

3.2.5 C-Rel is required for GM-CSF activation in T cells and macrophages

Given that c-Rel is regulated differently in the two cell types the requirement for c-Rel for *GM-CSF* transcription was determined using the c-Rel inhibitor pentoxifylline (PTX) (Wang et al. 1997). Cells were either left untreated or pre-treated with PTX for 30 minutes. Following pre-treatment cells were either left non-stimulated (NS) or stimulated for 4 hours with either PI (EL4 T cells) or LPS (RAW 264.7 macrophages).

In EL4 T cells a significant increase in *GM-CSF* gene expression was observed in the absence of c-Rel inhibition (Figure 3.8.A; $p<0.05$). In the presence of PTX this was significantly reduced (Figure 3.8.A; $p<0.05$) confirming that c-Rel is required for *GM-CSF* gene transcription in T cells. In RAW 264.7 cells an increase in *GM-CSF* mRNA levels is observed in the absence of PTX, although the amplitude of this response was variable (Figure 3.8.B; $p= 0.169$). In the presence of PTX a significant increase in *GM-CSF* mRNA levels is observed in response to LPS stimulation (Figure 3.8.B; $p<0.01$). The amplitude of this response is reduced, although not statistically significantly, due to the variation in the non-PTX treated control. These results suggest that whilst an increase in *GM-CSF* gene expression can be induced in the absence of c-Rel, c-Rel is required for higher levels of transcription. The effect of PTX on *GM-CSF* gene expression provides further support for a role for c-Rel in *GM-CSF* gene activation in T cells. However the inhibition of *GM-CSF* expression in PTX treated macrophages is not conclusive as c-Rel inhibition did not significantly reduce *GM-CSF* expression. Therefore further investigation was required to confirm that the divergence in c-Rel nuclear localisation observed in the western blot analysis accounts for the divergent CHX response observed between the two cell types.

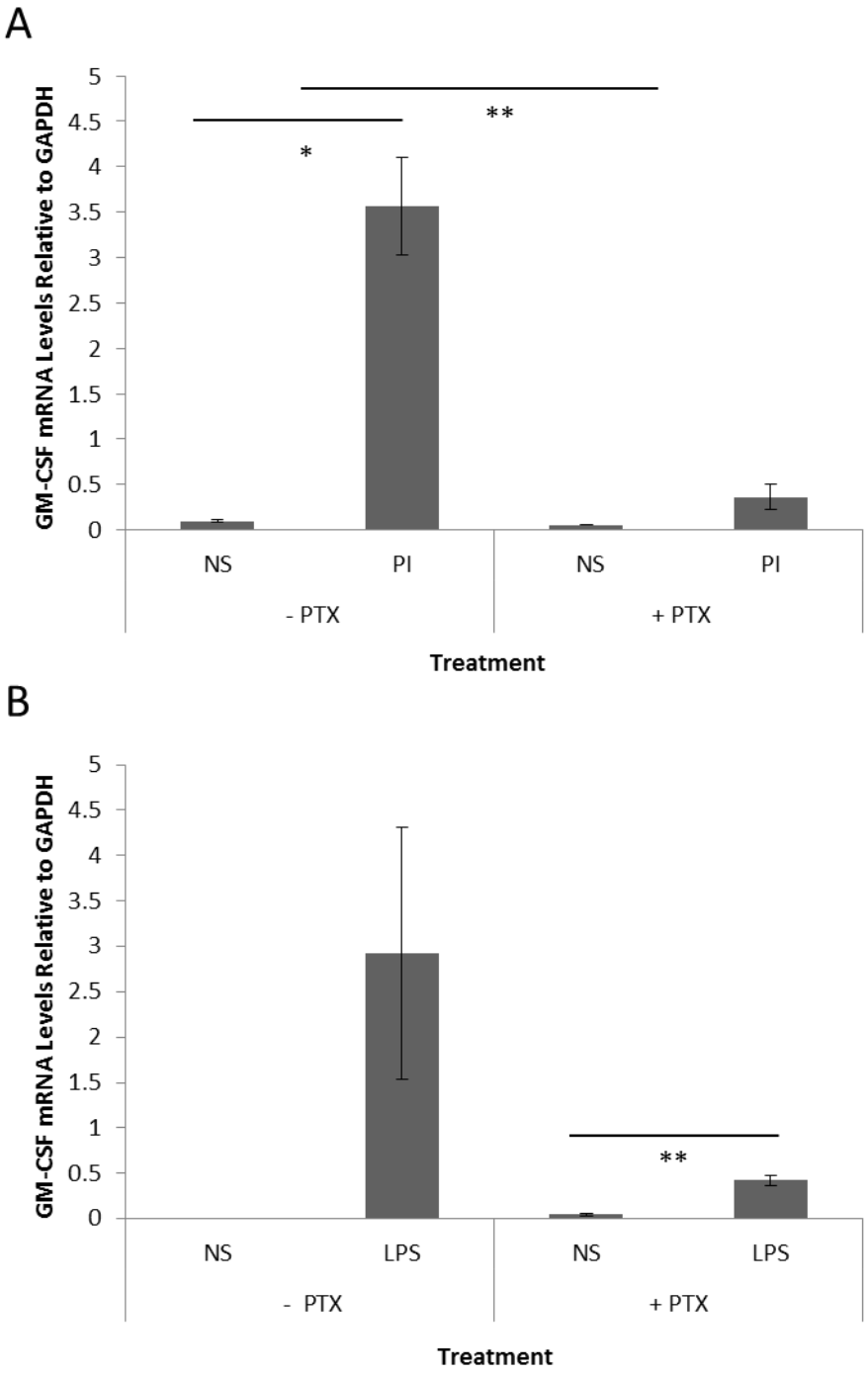

Figure 3.8: Pentoxifylline (PTX) inhibition of GM-CSF gene activation in T cells and macrophages. (A) Murine EL4 T cells were either left untreated or treated with PTX for 30 minutes before incubating with or without PI for 4 hours. (B) Murine RAW 264.7 macrophage cells were either left untreated or treated with PTX for 30 minutes before incubating with or without LPS for 4 hours. In each case, RNA was isolated and GM-CSF mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown. The significance from NS was determined by Students T. Test as was the significance of the change in gene expression between –PTX and +PTX treatments, * $p<0.05$. ** $p<0.01$.

To confirm these results, an alternative approach the role of c-Rel in activation of *GM-CSF* gene expression was examined in a knockout mouse model. CD4+ T cells and BMDM cells were purified from c-Rel$^{-/-}$ mice (Liou et al. 1999) and gene transcription responses examined in comparison to wild type C57/BL6 (WT) cells. T cells were co-stimulated with PI and CD28 which induced a significant increase in *GM-CSF* mRNA levels in WT cells (Figure 3.9.A; $p<0.05$). While an increase in *GM-CSF* mRNA levels was detected in c-Rel$^{-/-}$ T cells (Figure 3.9.A; $p= 0.121$), the gene response was significantly reduced compared to WT T cells ($p<0.05$), demonstrating that in the absence of c-Rel the inducible expression of *GM-CSF* is limited. This supports the hypothesis that c-Rel is an important transcription factor required for *GM-CSF* expression.

BMDM cells were treated with LPS to activate *GM-CSF* gene expression, which induced a high level of *GM-CSF* mRNA in WT cells (Figure 3.9.B), although this was highly variable and did not reach statistical significance ($p= 0.156$). BMDM derived from c-Rel$^{-/-}$ mice also produced increased levels of *GM-CSF* mRNA in response to LPS treatment (Figure 3.9.B; $p= <0.05$) but these levels were consistently lower than that which was observed in the WT cells. These data are consistent with what was found utilising PTX c-Rel inhibition and confirm that c-Rel has an integral role in *GM-CSF* gene transcription responses in both T cells and macrophages.

3.2.6 GM-CSF promoter chromatin accessibility

Association between c-Rel and the *GM-CSF* promoter has previously been shown to be required for chromatin remodelling across the previously defined promoter region in T cells (Brettingham-Moore et al. 2005; Poke et al. 2012). The requirement for chromatin remodelling complex assembly and activation prior to gene transcription events may explain the requirement for new protein synthesis prior to gene activation. Therefore changes in

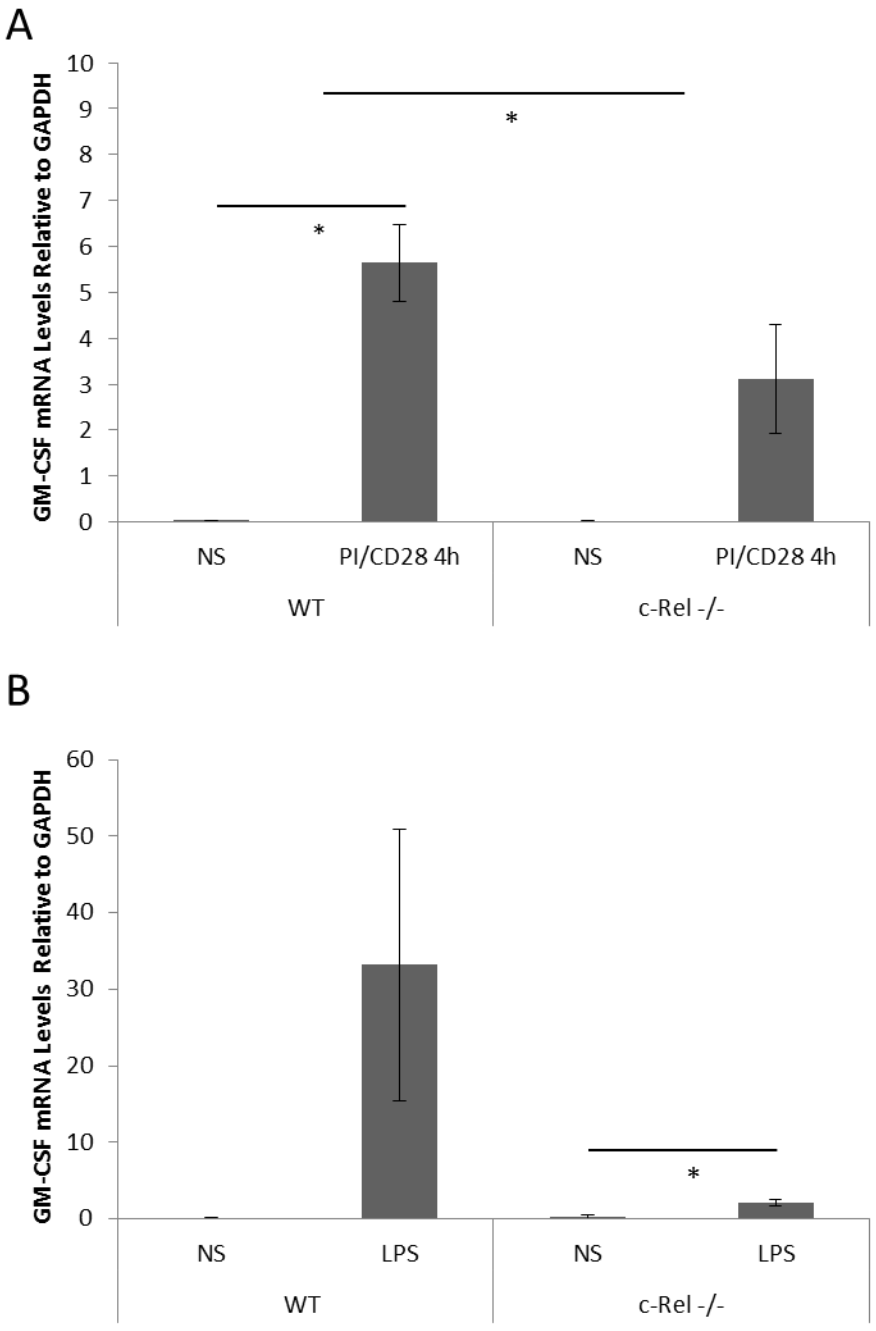

Figure 3.9: GM-CSF gene activation reliance on c-Rel; (A) CD4+ T cells were isolated from wild type C57/BL6 (WT) and c-Rel$^{-/-}$ mice. Cells were either non-stimulated (NS) or stimulated with PMA and calcium ionophore in conjunction with CD28 for 4hrs (PI/CD28). (B) Bone marrow derived macrophages were differentiated from WT and c-Rel $^{-/-}$ mice. Cells were either NS or stimulated with LPS for 4 hours. In each case, RNA was isolated and GM-CSF mRNA levels detected by RT-qPCR and adjusted to GAPDH. Mean and SEM of at least 3 replicates is shown. Significance from NS was determined by Students T. Test as was the significance of the change from NS between WT and c-Rel$^{-/-}$ cells, * $p<0.05$.

chromatin accessibility in response to stimulation were examined in the presence and absence of protein synthesis inhibition in T cells and macrophages using a previously described CHART-qPCR technique (Rao, Procko & Shannon 2001). This assay has previously been used in T cells to demonstrate that chromatin remodelling occurs across the *GM-CSF* promoter region in association with increased *GM-CSF* mRNA levels (Holloway et al. 2003).

In unstimulated T cells the promoter region is slightly accessible (uncut/cut ratio >1). Upon stimulation, EL4 T cells exhibit an increase in *GM-CSF* promoter accessibility to MNase1 (Figure 3.10.A) as has previously been described (Brettingham-Moore et al. 2005; Holloway et al. 2003). In the presence of CHX, this increase in accessibility is not observed (Figure 3.10.A). These results indicate that new protein synthesis is required to mediate the chromatin remodelling events which facilitate the increased promoter accessibility and subsequent GM-CSF transcription events.

In RAW 264.7 macrophage cells an increase in promoter accessibility is observed upon LPS stimulation (Figure 3.10.B; $p<0.05$) however the magnitude of this change is not as substantial as what was observed in T cells. In contrast to T cells, in the presence of CHX a significant increase in promoter accessibility of similar magnitude to that observed in the absence of CHX is induced ($p<0.05$). Importantly, these changes in accessibility also correlate with the changes in mRNA levels detected in RAW 264.7 cells (Figure 3.5.B).

In macrophage cells negative accessibility (uncut/cut value ratio <1, is detected in the unstimulated cells (Figure 3.10.B). This indicates that the non-digested samples contain less intact genomic DNA than those subjected to MNase1 digestion. It is conceivable that this may occur when the accessibility of the promoter is very close to zero as only at this level of accessibility would small errors in qPCR efficiency be unmasked and detectible as negative accessibility. Negative accessibility data has previously been published and reported as a mathematical artefact most likely attributable to error in

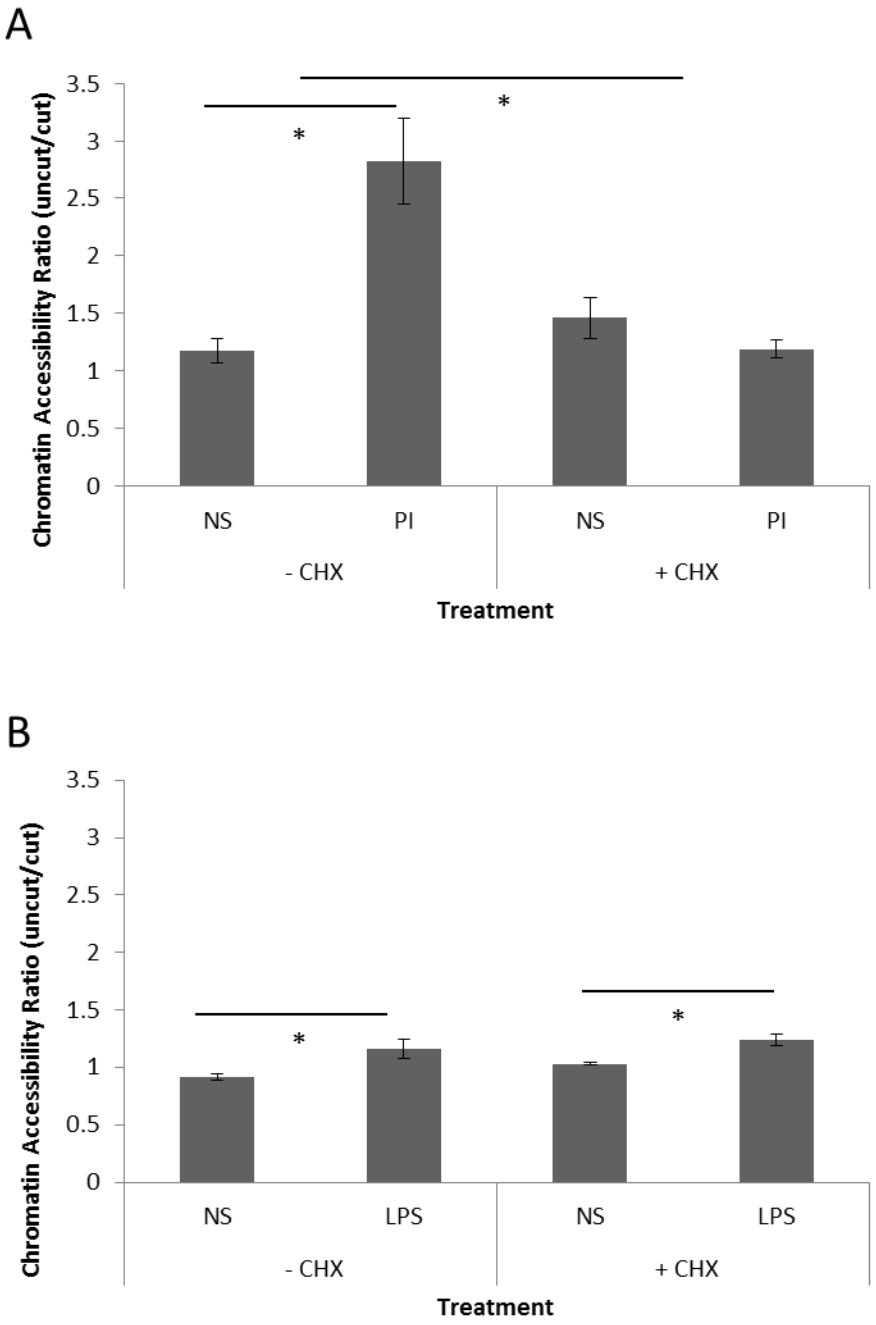

Figure 3.10: GM-CSF promoter accessibility patterns in macrophages compared to T cells. (A) Murine EL4 T cells were either left untreated or treated with CHX for 30 minutes before incubating with or without PI for 4 hours. (B) Murine RAW 264.7 cells were either left untreated or treated with CHX for 30 minutes before incubating with or without LPS for 4 hours. In each case, nuclei were digested with MNase1 and genomic DNA analysed by qPCR and compared to a non-digested control (uncut/cut). Mean and SEM of at least 3 replicates is shown. Significance from NS was determined in each treatment group as well as the change in accessibility between groups using a Students T. Test * $p<0.05$.

DNA quantitation and it was found to be neither reproducible nor biologically significant (Liang et al. 2006).

These data confirm that increased promoter accessibility occurs in conjunction with increased gene transcription. In EL4 T cells, treatment with PI for 4 hours results in an increase in *GM-CSF* promoter accessibility, but this increase is abolished by CHX treatment suggesting that chromatin remodelling events are reliant on newly synthesised proteins. In RAW 264.7 macrophage cells an increase in promoter accessibility is seen in response to LPS stimulation. Although significant, this increase was not as substantial as that observed in T cells, which correlates with the smaller increase in *GM-CSF* transcription observed in macrophages. Furthermore the increase in chromatin accessibility in macrophages occurs in the presence of CHX protein synthesis inhibition suggesting that the small change in chromatin accessibility observed in macrophage cells has different requirements to that observed in T cells.

3.2.7 Chromatin structure at the GM-CSF promoter

Basal chromatin structure established across gene regulatory regions has considerable influence over the activation requirements for gene expression. To further examine the changes in chromatin accessibility described above and with the purpose of determining if the *GM-CSF* promoter shares similar basal chromatin structure in T cells and macrophages, chromatin immunoprecipitation assays (ChIP) were performed. Association between the core histone protein H3 and H3 acetylation with the upstream *GM-CSF* enhancer, a region 1.1 kb 5' of the *GM-CSF* promoter and the inactive *rhodopsin* promoter were examined alongside the *GM-CSF* promoter and have been reported previously in T cells (Poke et al. 2012).

Core histone protein H3 was examined to give an estimation of the relative nucleosome occupancy of the DNA region of interest and to determine if this is reflective of the

relative chromatin accessibility. In non-stimulated EL4 T cells the *GM-CSF* promoter region shows a similar level of H3 association to that of the *GM-CSF* enhancer and the region 1.1 kb 5' (Figure 3.11.A). Following 4 hours of PI stimulation, H3 associated with the *GM-CSF* promoter is depleted ($p<0.05$) whilst no significant loss of H3 is observed from the control regions (Figure 3.11.A). This loss of H3 is indicative of chromatin remodelling events occurring at the promoter region (Figure 3.10.A).

Regions of chromatin which undergo chromatin remodelling events are frequently marked by chromatin modifications such as histone acetylation which may be recognised by further modifying enzymes as well as chromatin remodelling complexes (Dhalluin et al. 1999). In T cells, the *GM-CSF* promoter is specifically marked by higher levels of H3 acetylation than the control regions (Figure 3.11.B). This acetylation is lost upon 4 hours of PI stimulation ($p<0.05$) which correlates with the loss of core H3 proteins from the promoter (Figure 3.11.A). Notably the levels of H3 acetylation detected at the inactive *rhodopsin* promoter region are markedly lower and do not vary with stimulation as is consistent with inactive chromatin structure.

In non-stimulated RAW 264.7 macrophage cells the *GM-CSF* promoter displays a similar level of core histone protein H3 to the *GM-CSF* enhancer and control region as well as to the distal inactive *rhodopsin* promoter (Figure 3.12.A). Upon LPS stimulation a decrease in H3 association is observed although this is not significant and a comparable loss in H3 is seen across all of the control regions including the *rhodopsin* promoter (Figure 3.12.A). These data suggest that there is no significant depletion of H3 from the promoter region in macrophages such as is observed in T cells. In macrophage cells the GM-CSF promoter H3 acetylation levels are comparable to those is detected at the inactive *rhodopsin* promoter (Figure 3.12.B) indicating that the promoter is not marked by higher acetylation levels. In addition no change in H3 acetylation is observed at any of the regions examined in response to LPS stimulation. Therefore, it would appear that the *GM-CSF* promoter is not marked for activation by H3 acetylation in macrophages as it is in T cells and in addition any chromatin

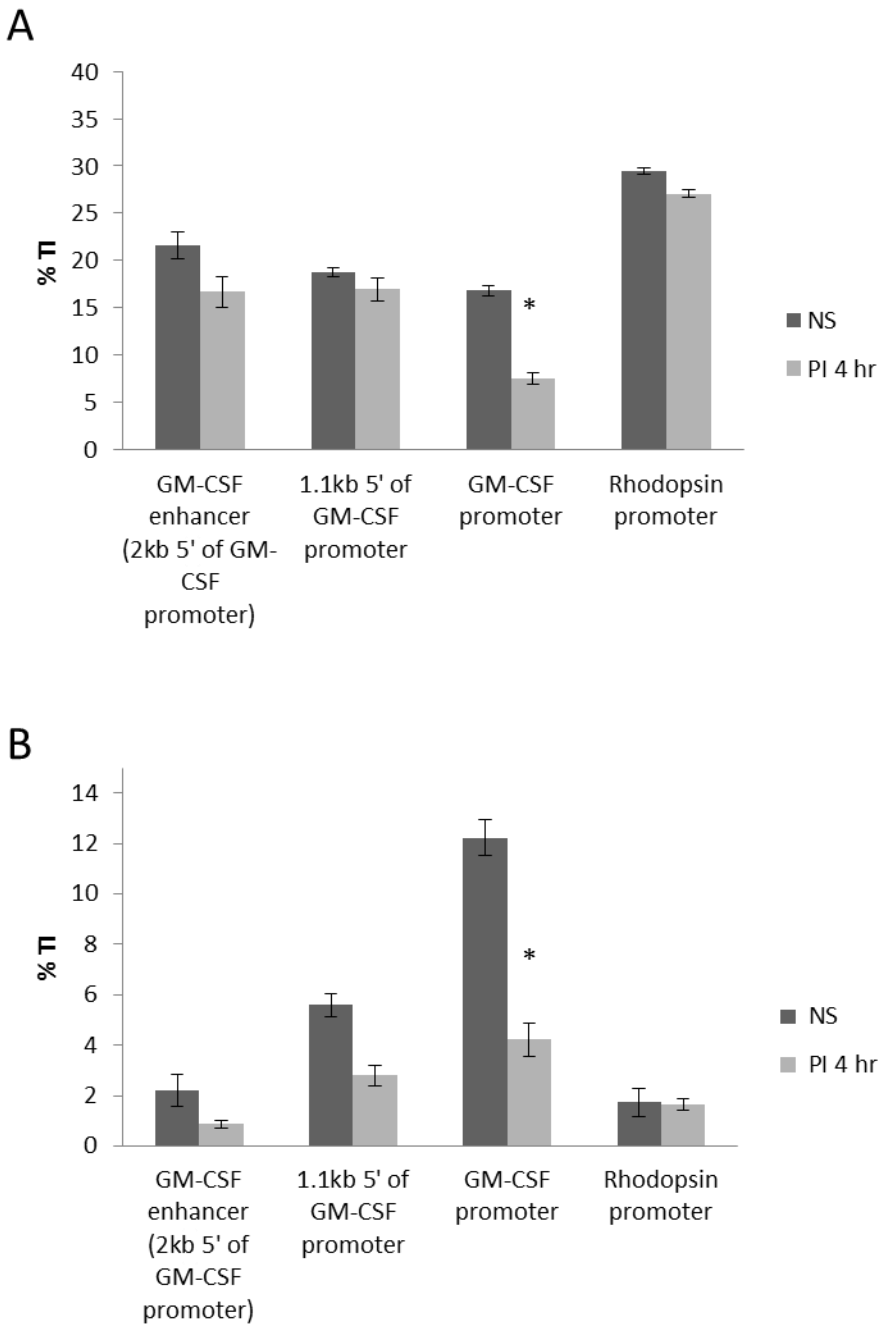

Figure 3.11: Histone occupancy and acetylation at the GM-CSF promoter in EL4 T cells. Chromatin immunoprecipitation (ChIP) performed on EL4 T cells which were either non-stimulated (NS) or stimulated with PI for 4 hours (PI). Antibodies were used against core histone protein H3 (A) and acetyl H3 (B). Enrichment of the proteins across the GM-CSF gene and the inactive rhodopsin promoter was analysed by qPCR and normalised to total input (%TI). Mean and SEM of at least 3 replicates is shown. Significance from NS was determined by Student T. Test * $p<0.05$. (Previously published data: Poke et al. 2012).

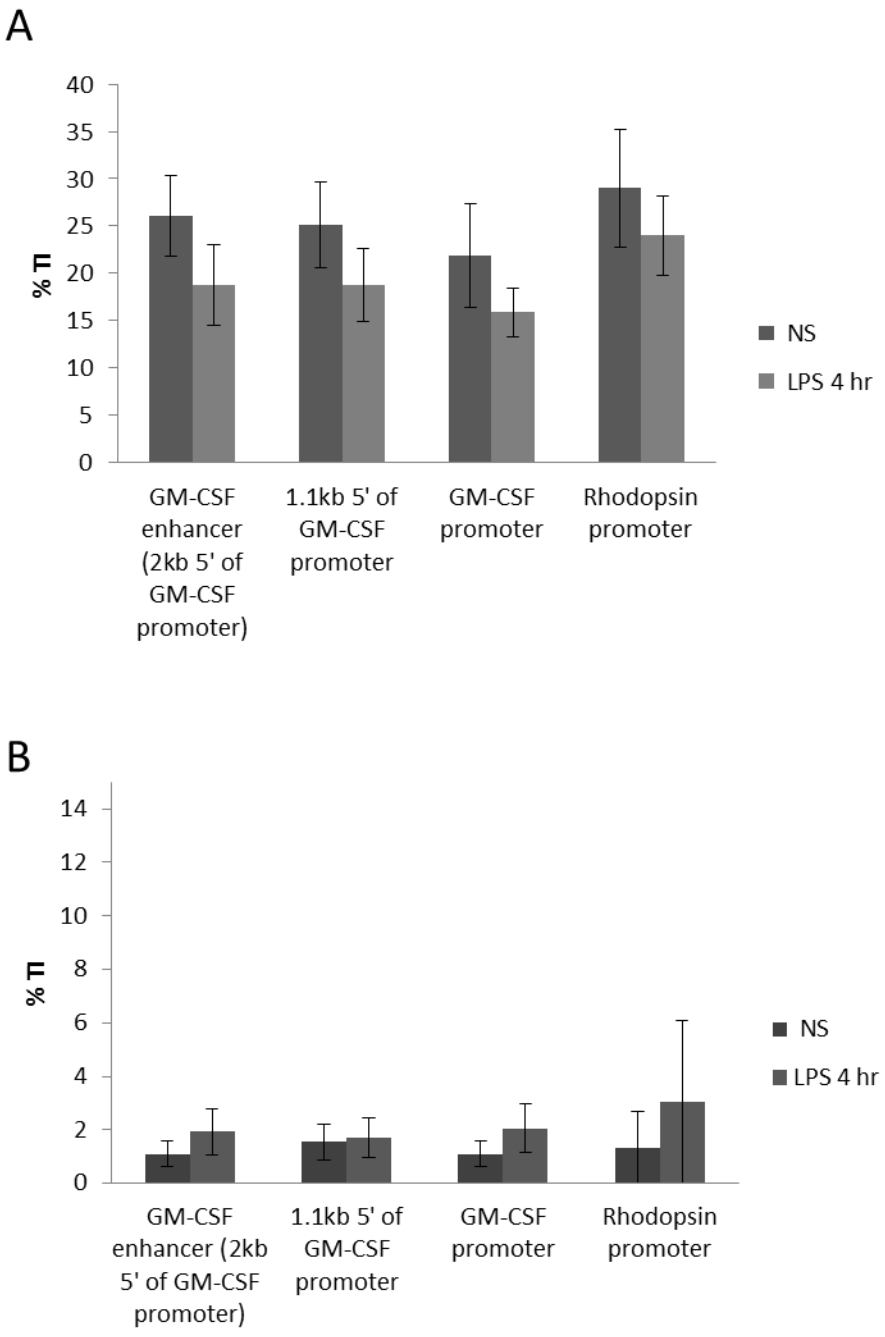

Figure 3.12: Histone occupancy and acetylation at the GM-CSF promoter in macrophages. Chromatin immunoprecipitation (ChIP) performed on RAW 264.7 macrophage cells which were either non-stimulated (NS) or stimulated with LPS for 4 hours (LPS). Antibodies were used against core histone protein H3 (A) and acetyl H3 (B). Enrichment of the proteins across the GM-CSF gene and the inactive rhodopsin promoter was analysed by qPCR and normalised to total input (%T.I). Mean and SEM of at least 3 replicates is shown. No significant difference from NS was determined by Student T. Test.

remodelling events that are observed in association with *GM-CSF* activation in macrophage cells are brought about independent of H3 loss.

These results independently confirm the changes in chromatin structure at the promoter region observed in the chromatin accessibility assays. In T cells significant loss of acetylated H3 proteins from the promoter region results in an increase in promoter accessibility which is permissive for increased gene transcription. In macrophages this loss of H3 was not observed in response to stimulation. This is reflective of the small increase in promoter accessibility and gene transcription triggered by stimulation. In addition the promoter region is not marked by histone acetylation indicating that the basal chromatin environment established at the *GM-CSF* promoter is different in T cells compared to macrophages.

3.3 Discussion

The data presented here demonstrates that immune genes may be expressed in multiple cell types and display similar activation kinetics, however the mechanisms regulating gene activation can be fundamentally different in the divergent cell types. While the *GM-CSF* gene is activated with similar kinetics in T cells and macrophages, this appears to involve two clear mechanistic differences. Firstly, in T cells the *GM-CSF* gene is classified as a secondary response gene based on its reliance on new protein synthesis, whereas in macrophages *GM-CSF* is a delayed primary response gene with increased expression independent of new protein synthesis. While the data presented clearly shows that c-Rel is required for *GM-CSF* transcription in both cell types, the divergence in response type may be partially explained by a difference in the requirement for protein synthesis for c-Rel nuclear localisation in the two cell types. Secondly, the chromatin remodelling events which increase accessibility of the *GM-CSF* promoter in response to stimulation, and accompany transcriptional activation of the gene, occur by fundamentally different mechanisms in T cells compared to macrophages.

3.3.1 Protein synthesis requirements – Primary versus secondary responses

The requirement for new protein synthesis for increased gene transcription has widely been used to classify genes as primary and secondary response genes (Fowler, Sen & Roy 2011; Hargreaves, Horng & Medzhitov 2009; Yamamoto & Alberts 1976). These differing requirements have previously been found to reflect differences in activation kinetics. However the data presented in this chapter revealed that despite having similar activation kinetics, in T cells *GM-CSF* is categorised as a secondary response gene

reliant on de novo protein synthesis for increased gene transcription whereas in macrophages *GM-CSF* expression is independent of new protein synthesis, as previously documented (Brettingham-Moore et al. 2005; Ramirez-Carrozzi et al. 2009).

The data presented here shows that, not only is gene transcription permissible in the presence of CHX in macrophage cells, the amplitude of mRNA transcript levels was in fact elevated. Enhanced mRNA levels in response to CHX treatment have previously been reported for other inducible immune genes (Roger, Out, Jansen, et al. 1998). This is conceivably due to the non-specific nature of blocking translation or a regulatory mechanism in response to transcription initiation signals but no resultant translation of proteins occurring. Plausibly this enhanced expression may be due to off-target effects of CHX blocking the synthesis of inhibitory proteins which down regulate gene expression. However, this phenomenon, termed superinduction, has previously been reported for other genes and has largely been attributed to enhanced mRNA stability in the presence of CHX. Enhanced *IL-6* mRNA stability has previously been examined by inhibiting transcription of new mRNA through treatment of cells with actinomycin. mRNA half-life was then determined by northern blot analysis of RNA at a number of different time points (Roger, Out, Jansen, et al. 1998; Roger, Out, Mukaida, et al. 1998). This supports the notion that no new synthesis of transcriptional activators is required for increased gene transcription to occur, confirming the previous classification of *GM-CSF* as a primary response gene i.e. independent of CHX in macrophages (Ramirez-Carrozzi et al. 2009).

Interestingly, when the *GM-CSF* transcriptional response was examined in murine embryonic fibroblasts (MEFs), the superinduction response in the presence of CHX is also observed. This raises the possibility that the divergence in CHX response may be stimulus driven as both the MEF cells and macrophages were stimulated with LPS via the TLR4 MyD88 signalling pathway. In T cells increased mRNA transcription was triggered by directly activating the protein kinase C and calcium signalling pathways downstream of the TCR through treatment of cells with PMA and calcium ionophore. The murine EL4 T cell line is unable to respond to LPS as it does not have the

appropriate intact cell surface receptors and the murine RAW 264.7 cell line did not produce a discernible increase in *GM-CSF* expression in response to PI stimulation. Therefore this divergence was unable to be examined by using the same stimuli for both cell types. LPS is routinely used to activate macrophage cells and has previously been reported to be a potent activator of *GM-CSF* (Li et al. 2012; Thorens, Mermod & Vassalli 1987) in addition to the other macrophage growth and differentiation factor, macrophage colony stimulating factor (M-CSF) (Becker, Devlin & Haskill 1989). Signalling through TLR4 activates a series of downstream signalling cascades including the protein kinase C pathway that is activated by PMA (Sweet & Hume 1996). However additional signalling events must be required as LPS was able to trigger increased expression of *GM-CSF* whereas PI stimulation was not.

Whilst the different stimulus and signalling cascade is worthy of consideration, it is unlikely that this accounts for all of the difference in response between cell types. Activation of naïve T cells results in the slow differentiation into cytokine producing effector cells, whereas stimulation via the same cell surface signalling events in memory T cells triggers much more rapid cytokine expression suggesting that even though initial signalling events are the same, distinct regulatory mechanisms can exist downstream of the activation signals (Cuddapah, Barski & Zhao 2010). Furthermore, different populations of macrophages produce different activation profiles for immune genes, including for *GM-CSF*, in response to the same stimuli (Grigoriadis et al. 1996). This suggests that specific populations of cells are able to respond to the same stimuli in different ways in order to produce a targeted immune response.

Both the PKC and TLR4 signalling pathways trigger activation of NF-κB proteins (Figure 1.1). Data from this study suggest that c-Rel nuclear activation has different requirements for protein synthesis in T cells compared to macrophages. Western blot analysis showed that c-Rel nuclear translocation is inhibited by CHX treatment in T cells but is still permissive in CHX treated macrophage cells suggesting that a divergence in c-Rel activation may account for the divergence in CHX response. c-Rel was shown to undergo translocation from the cytoplasm to the nucleus in response to PI

stimulation in T cells and LPS stimulation in macrophages. This increase in c-Rel levels in the nucleus is not detected in the presence of CHX in T cells and increased synthesis of c-Rel in the cytoplasmic fraction is abolished suggesting that CHX inhibits c-Rel synthesis and activation. In contrast, in macrophage cells an increase in c-Rel nuclear localisation in response to LPS is sustained in the presence of CHX. Additionally CHX treatment alone appears to enhance nuclear localisation of c-Rel in macrophage cells. Enhanced c-Rel nuclear localisation in the presence of protein synthesis inhibition suggests that in macrophage cells the continued synthesis of a c-Rel inhibitory molecule is required to maintain cytosolic localisation. In contrast fellow NF-κB family transcription factor, Rel-A is constitutively expressed and does not display evidence of inhibition in the presence of CHX in T cells. These results further suggest that the divergence in CHX dependence is due to divergence in c-Rel regulation, which may be either cell type or stimulus specific.

The role of c-Rel in mediating *GM-CSF* gene responses was further confirmed with the pharmacological c-Rel inhibitor, pentoxifylline (PTX) as well as in cells derived from c-Rel$^{-/-}$ mice. Results from both of these models demonstrate a critical role for c-Rel in *GM-CSF* activation in both T cells and macrophages.

3.3.2 Chromatin structure and remodelling

Data presented in this chapter suggest that both the basal chromatin structure established across the *GM-CSF* promoter and remodelling events in response to cell activation differ between T cells and macrophages. Firstly, prior to activation, the promoter region is marked by high levels of histone acetylation in T cells but not macrophages. Additionally, following T cell activation significant loss of acetylated H3 proteins from the *GM-CSF* promoter region results in an increase in promoter accessibility which is permissive for increased gene transcription. In contrast, in macrophages no such loss of H3 was observed in response to stimulation. In addition only a small increase in accessibility was observed at the promoter region in stimulated

macrophages. As changes in chromatin accessibility reflective of chromatin remodelling are often associated with increased gene transcription (Cockerill 2011) this may reflect of the smaller increase in *GM-CSF* gene transcription triggered by stimulation in macrophages compared to T cells. Previous work has identified c-Rel as an important regulator for chromatin remodelling events which occur in T cells at the *GM-CSF* promoter to permit increased gene transcription (Brettingham-Moore et al. 2005).

The basal chromatin state at the GM-CSF promoter appears to be different between the two cell types examined. This is likely to be established during the process of cell differentiation. In early T cell development in the thymus, the *IL-3*/*GM-CSF* locus is retained in an epigenetically silent state marked by repressive histone modifications (Mirabella et al. 2010). Chromatin mediated repression of potent pro-inflammatory cytokines assists in preventing inappropriate inflammatory responses. Effectively *GM-CSF* gene transcription in response to activation of the PKC and calcium signalling pathway is abolished (Mirabella et al. 2010). During the process of differentiation into mature T cell lineages, cells progressively acquire active chromatin modifications and gene inducibility increases (Mirabella et al. 2010). This suggests that chromatin structures established during differentiation from a common precursor can dictate the transcriptional response of the *GM-CSF* gene. In T cells, *GM-CSF* gene activation occurs in association with BRG1 mediated nucleosome remodelling (Brettingham-Moore et al. 2008). Evidence of this is seen in the loss of highly acetylated H3 histones from the *GM-CSF* promoter region in response to PI stimulation. Histone acetylation marks regions of DNA for subsequent gene expression and is able to recruit chromatin remodelling complexes (Hassan et al. 2001) so may explain how the chromatin established at the *GM-CSF* promoter primes the gene for subsequent inducible gene expression.

In macrophage cells a similar level of basal H3 occupancy (about 20% T.I) is detected at the *GM-CSF* promoter however the promoter region is not marked by high levels of acetylation and no significant loss of histone protein H3 was seen upon LPS

stimulation. This may be a reflection of the difference in magnitude of the transcriptional response induced by LPS in macrophage cells compared to PI stimulation of T cells, or may indicate that only a small population of the macrophage cells are responding to stimuli compared to the T cells. It is probable that a more significant increase in gene expression and also a greater increase in chromatin accessibility may be induced with increasing concentrations of LPS as it has previously been reported that LPS is internalised rapidly in macrophages and rapidly broken down enzymatically (Sweet & Hume 1996; Ulevitch & Tobias 1995). However *E. coli* derived LPS has been used at only 1μg/mL to induce GM-CSF expression in RAW 264.7 macrophages (Li et al. 2012). The type of LPS used throughout these experiments has previously been shown to induce immune gene expression in the J774 macrophage cell line at 10μg/mL, at the same concentration used throughout these experiments (Ramirez-Carrozzi et al. 2009).

In T cells a significant increase in promoter accessibility was seen in response to stimulation which is reflective of the loss of nucleosome proteins from this region. This response is significantly reduced in the presence of CHX suggesting that the synthesis of new proteins is required for chromatin remodelling events at the *GM-CSF* promoter in T cells. In contrast, in macrophages a small increase in promoter accessibility is observed in response to LPS stimulation despite no evidence of significant loss of H3 proteins. This increase in accessibility in response to stimulation was observed both in the presence and absence of CHX, indicating that no new protein synthesis is required to enable this level of chromatin remodelling. If c-Rel is required to mediate chromatin remodelling events in macrophages as it is in T cells then the increased nuclear localisation of c-Rel in CHX treated macrophages may explain why chromatin remodelling is permissible in CHX treated macrophages. Alternatively it may indicate that the low level chromatin remodelling may take place utilising an alternative mechanism in macrophage cells. The lower level of increased accessibility in macrophage cells appears to occur without significant loss of H3 proteins from the promoter region. This may be due to only partial disassembly/sliding of the

nucleosome, which may utilise a Mi2 or ISWI family chromatin remodeller as opposed to the SWI/SNF BRG1 remodelling enzyme utilised in T cells.

3.3.3 Conclusions

The data presented in this chapter shows that in T cells the GM-CSF gene is a secondary response gene marked by high levels of histone acetylation. Upon stimulation an increase in promoter accessibility is facilitated by loss of core histone protein H3 from the promoter region. This increase in accessibility enables high levels of gene transcription to take place. This chromatin remodelling and associated gene transcription requires new protein synthesis. Despite having similar activation kinetics in macrophages, *GM-CSF* expression is independent of new protein synthesis and therefore classified as a delayed primary response gene. Only a small change in promoter accessibility is observed in association with increased gene transcription and does not involve significant loss of H3 from the promoter region. This suggests that lower levels of transcription may be permissible without significant chromatin remodelling. Previously it has been shown that treatment of T cells with ionophore alone triggers low level *GM-CSF* transcription without chromatin remodelling events (Brettingham-Moore et al. 2005). Additionally the promoter region is not marked for remodelling by high levels of histone acetylation in macrophages as it is in T cells.

Despite divergence in the activation requirements between the two cell types the NF-κB transcription factor c-Rel is shown to be required for *GM-CSF* expression in both cell types. These results highlight that the interaction between basal chromatin environment, transcription factor availability and chromatin remodelling events are important for the inducible activation of immune genes, and together allow genes to respond to immune signals by different mechanisms in different cell types.

4 Inducible cytokine gene expression

4.1 Introduction

The role of chromatin architecture established during the process of differentiation in mediating lineage commitment and mature cell type capabilities is increasingly being appreciated (Cedar & Bergman 2011; Falvo et al. 2013; Georgopoulos 2002; Zhang et al. 2012). In fact, the transcription factors involved in mediating cell fate do so not only through the recruitment of the transcription machinery to transcriptional start sites but also through interactions with chromatin modifying complexes that are important for establishing lineage specific chromatin architecture (Laiosa, Stadtfeld & Graf 2006; Vahedi et al. 2012). This raises the possibility that genes which are expressed in multiple cell types utilising similar transcription factors may produce a unique response due to the different chromatin architecture established at that gene during the process of differentiation (Smale 2010a).

Genome-wide studies utilising technologies such as ChIP-on-chip and ChIP-seq have rapidly advanced our understanding of how chromatin landscapes are associated with gene regulation. We now have an understanding of typical patterns of histone modification which are associated with gene promoters, enhancers, gene bodies and transcribed regions (Barski et al. 2007; Lim et al. 2009; Lim et al. 2013). High levels of H3 and H4 acetylation as well as H3K4 methylation are detected at promoter regions of actively transcribed genes (Roh et al. 2006; Roh, Cuddapah & Zhao 2005; Saccani, Pantano & Natoli 2001). Additionally, mono-methylation of H3K27, H3K9, H4K20, H3K79 and H2BK79 are linked to gene activation (Barski et al. 2007). In contrast, tri-methylation of H3K27, H3K9 and H3K79 are linked to repressed genes (Barski et al.

2007; Roh et al. 2006). These histone modifications are dynamically added to through the recruitment and activity of histone–modifying enzymes (Lim et al. 2013). Genome-wide mapping of histone acetyltransferases (HATs) and deacetylases (HDACs) has revealed several categories of HAT/HDAC activity which correlate with gene expression. HDACs are recruited not only to repressed genes but are also highly associated with active genes, where both HATs and HDACs have a high level of activity associated with RNA polymerase binding and gene transcription (Wang et al. 2009). Genes that are primed for subsequent gene activation events are not necessarily marked by high levels of acetylation but both HATs and HDACs appear to bind transiently suggesting that acetyl groups are actively added and removed frequently from primed promoters ready for rapid activation (Wang et al. 2009). Furthermore the incorporation of histone variants such as H2A.Z in place of canonical histone proteins occurs at regulatory elements of DNA (Barski et al. 2007). Whilst these studies have been pivotal to understanding how epigenetic markers are distributed and elucidate general rules which govern gene regulation, the intricate interaction between different regulatory components at gene specific sites is often overlooked. These include how individual transcription factors interact with the chromatin environment.

Despite early divergence in the haematopoietic pathway and functionally distinct roles, CD4+ T lymphocyte cells and macrophages must coordinate and initiate immune responses in a cooperative manner. As such, cells of the divergent lineages retain the ability to induce the transcription of a number of shared immune modulating cytokine genes in addition to the *GM-CSF* gene which was discussed in Chapter 3. The *GM-CSF* gene has been well characterised in T cells and its inducible gene transcription is documented as being dependent on the synthesis of new protein, the transcription factor c-Rel and the chromatin remodeller BRG1, as discussed in Chapter 3 and described elsewhere (Brettingham-Moore et al. 2005; Brettingham-Moore et al. 2008; Poke et al. 2012).

Both BRG1 and c-Rel have previously been identified as playing a significant role in immune gene regulation in macrophage cells also (Grigoriadis et al. 1996; Ramirez-

Carrozzi et al. 2006; Schreiber et al. 2006). A study coupling ChIP for NF-κB transcription factors with microarray analysis of gene expression determined that, in human monocytic cells c-Rel binds 83 gene promoters in response to LPS stimuli, only 10% of which were occupied prior to stimulation (Schreiber et al. 2006). This suggests an important role for c-Rel in regulating inducible genes. From gene expression analysis in cells isolated from c-Rel$^{-/-}$ mice, c-Rel can be inferred as serving distinctly unique roles in regulating gene expression in different mature macrophage populations. Hematopoietic precursors in c-Rel$^{-/-}$ mice retain the ability to differentiate into mature macrophages. However the normal mature macrophage population is somewhat heterogeneous and different populations of mature macrophages isolated from c-Rel$^{-/-}$ mice exhibit defective cytotoxic killing, nitric oxide production as well as altered expression of certain cytokines including *GM-CSF* (Grigoriadis et al. 1996) suggesting that c-Rel may play a different regulatory role in different macrophage populations.

The *GM-CSF* gene is c-Rel dependent in both T cells and macrophages (Chapter 3) but switches from being a BRG1 dependent gene in T cells to being independent of SWI/SNF in macrophages (Grigoriadis et al. 1996; Poke et al. 2012; Ramirez-Carrozzi et al. 2009). In addition, there is a switch in the requirement for new protein synthesis for gene transcription between the two cell types as is discernible by gene expression analysis in the presence of the translation inhibitor, cycloheximide (CHX). Genome-wide data provides a valuable resource in which to identify other cytokine and immune modulating genes which are regulated by c-Rel in both T cells and macrophages and determine if these are common features of c-Rel dependent gene expression in the two cell types.

The aim of this chapter was to utilise genome-wide data to identify the cohort of c-Rel dependent genes which have inducible expression in both T cells and macrophages, and identify genes which share similar activation requirements to *GM-CSF*. Further, the aim of this chapter was to determine whether c-Rel dependent genes are regulated by fundamentally different mechanisms in the two cell types.

4.2 Results

4.2.1 Inducible expression of potential BRG1 and c-Rel regulated genes

In order to identify additional inducible immune genes that, like the *GM-CSF* gene, require BRG1 and c-Rel for gene activation in T cells and are also expressed in macrophage cells, bioinformatic analysis of available genome-wide data was performed in conjunction with Dr. Kristine Hardy (*Faculty of Education, Science, Technology and Mathematics, The University of Canberra, Canberra, ACT, Australia).* The strategy employed is outlined in Figure 4.1. Genome-wide c-Rel dependence was determined by analysis of Affymetrix gene expression array data generated by G. Chen (*JCSMR, ANU*) from wild-type and c-Rel$^{-/-}$ CD4+ splenocytes, either unstimulated or stimulated with PMA and calcium ionophore (PI) for 8 hours (G Chen, K Hardy and MF Shannon, unpublished). Analysis of this data identified 268 genes which showed increased gene expression in response to PI stimulation in the WT cells compared to the c-Rel$^{-/-}$ cells with a $\geq \text{Log}_2$ 0.5 difference. These genes were classified as c-Rel-dependent genes.

To determine which of the c-Rel dependent cohort were likely to be BRG1 regulated, publically available ChIP-seq data was analysed (De et al. 2011). This data set included BRG1 ChIP-seq analysis of naïve T cells and also Th1, Th2 and Th17 cells before and after activation (De et al. 2011). Analysis of these data sets, revealed that approximately 60% of c-Rel dependent genes in CD4+ T cells bind BRG1, with 23% displaying basal BRG1 binding within 1kb of their transcription start site, and 38% demonstrating enhanced BRG1 binding in stimulated T cells. These values are similar to other inducible genes, but much higher than for all genes on the array, suggesting as expected that BRG1 plays an important role in the regulation of inducible genes. Of the 101 genes that gain BRG1 binding in activated cells, 72 have NF-κB motifs within 200 bp

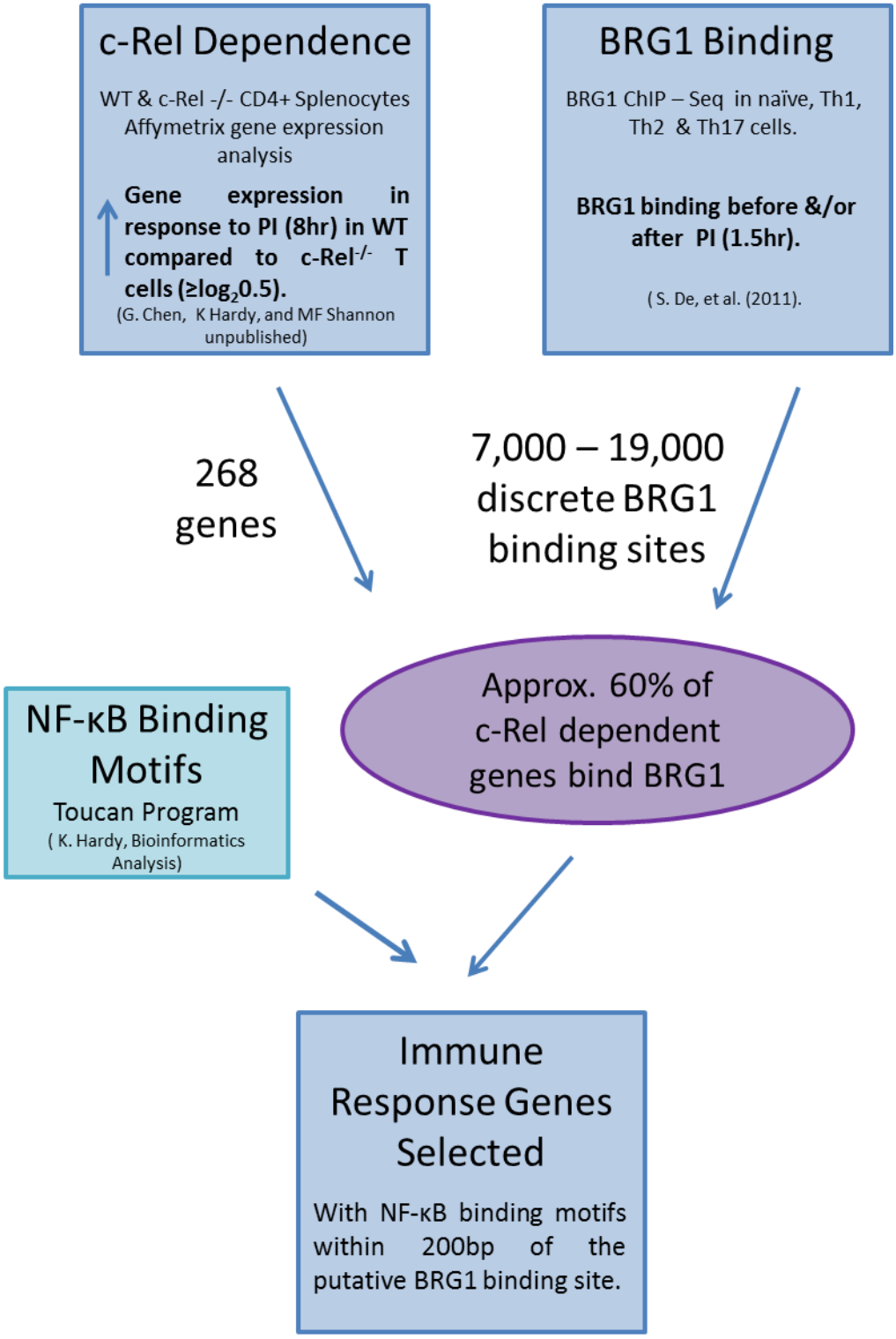

Figure 4.1: Summary of the bioinformatic strategy used to identify potential c-Rel and BRG1 dependent genes.

of the BRG1 binding site, as identified using the Toucan program. This list represents genes that are potentially regulated by both the chromatin remodeller BRG1 and NF-κB transcription factors, including c-Rel. A set of c-Rel regulated genes which bind BRG1 and a set of genes which did not bind BRG1 in this data set were selected for further analysis to determine if common features distinguish these two gene sets.

The BRG1 independent genes, *IL-6*, *IL-10*, *IL-1a* and *IL-22* were selected for further analysis, as were the BRG1-dependent genes, *IL-23A*, *IL-21*, *Tnfsf9* and *IFN-γ* due to their respective roles in immune regulation. Expression of these genes was initially examined by RT-qPCR in primary CD4+ T cells either left untreated or treated with PI for 4 hours to confirm inducible expression in T cells. All genes examined showed very low or no basal mRNA expression and increased mRNA levels in response to PI stimulation (Figure 4.2). To determine if these genes were also expressed in macrophage cells so that regulatory mechanisms of inducible gene expression could be examined in the two cell types, gene expression analysis was also performed in bone marrow derived macrophages (BMDM) in response to LPS. As in T cells, no or low basal mRNA expression was observed, and in response to LPS stimulation an increase in mRNA levels was observed for all genes with the exception of *IL-21* and *IL-22* (Figure 4.3). This indicates that, as expected, not all immune genes are expressed in both cell types. Whilst the majority of genes showed evidence of up regulation in response to stimulation, the extent of this increase was variable. *GM-CSF* ($p<0.05$) and *IFN-γ* ($p<0.05$) displayed the highest levels of expression in T cells (Figure 4.2), whilst other genes such as *IL-6* ($p= 0.122$) showed only a small fold change in gene expression (Figure 4.2). In macrophage cells, *IL-1a* showed the highest level of expression ($p<0.05$), whilst *GM-CSF* also showed comparatively high levels of inducible gene expression ($p<0.01$).

These results show that a number of immune genes are inducibly expressed in both T cells and macrophages in response to appropriate stimuli and may potentially share activation requirements with the *GM-CSF* gene.

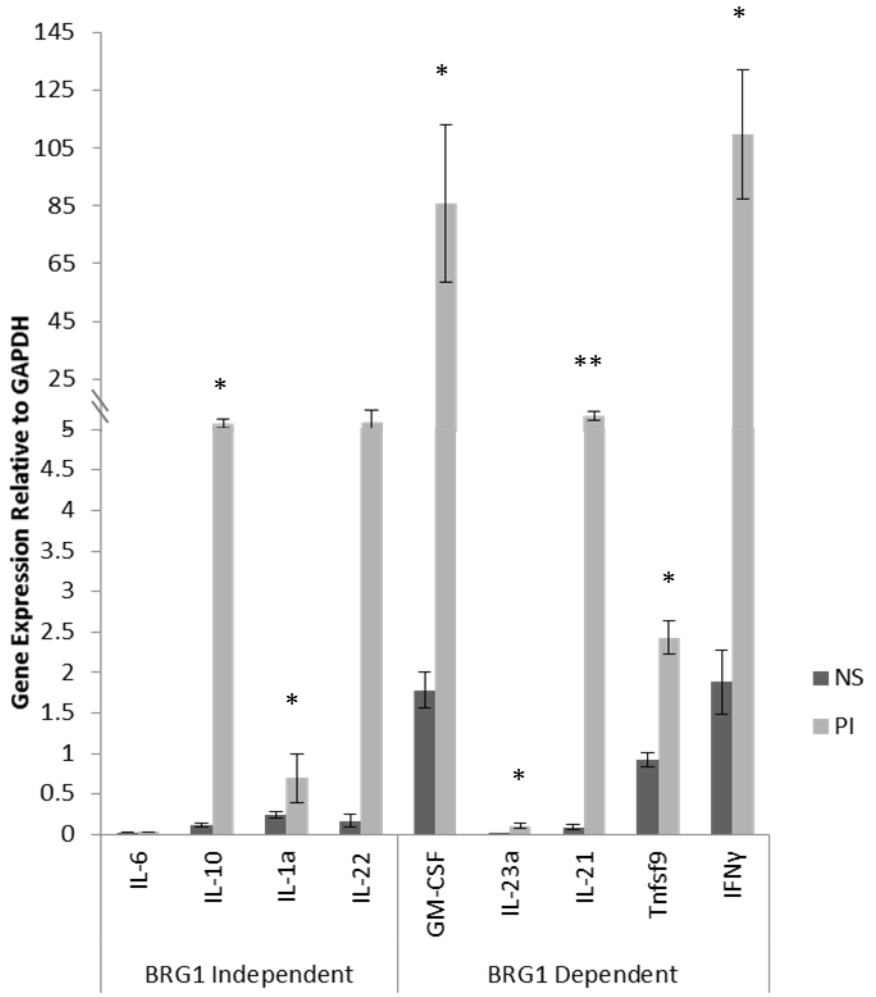

Figure 4.2: Inducible immune gene expression in primary CD4+ T cells. CD4+ T cells were isolated from C57/BL6 mice and either left non-stimulated (NS) or treated with PMA and calcium ionophore (PI) for 4 hours. RNA was isolated and mRNA levels were determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown. Significance from NS was determined by Student T. Test, * $p<0.05$. ** $p<0.01$.

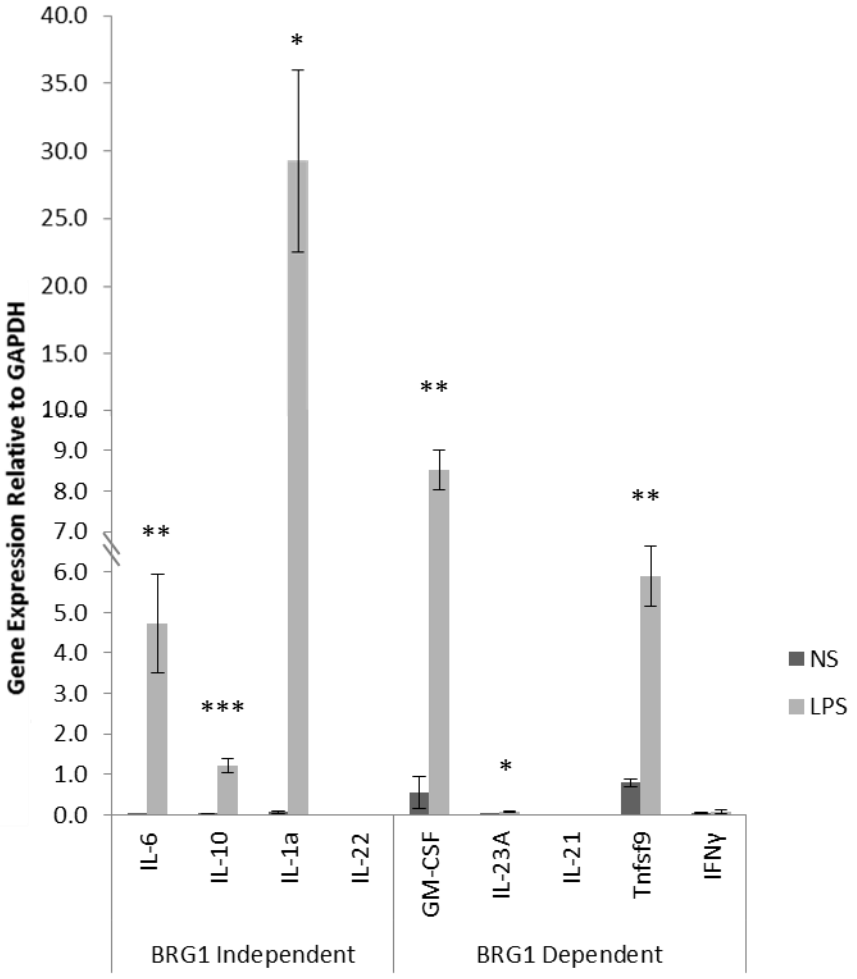

Figure 4.3: Inducible gene expression in primary bone marrow derived macrophages. BMDM cells were differentiated from bone marrow extracted from C57/BL6 mice and either left non-stimulated (NS) or treated with LPS for 4 hours. RNA was isolated and mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown. Significance from NS was determined by Students T. Test * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.2.2 Protein synthesis dependence of inducible gene sets

Evidence presented in Chapter 3 indicates that *GM-CSF* gene induction switches between being inhibited in the presence of CHX in T cells to being CHX independent in macrophages. This raises the possibility that this "switching" may represent a mechanism employed by other inducible genes. To determine if this switch in activation requirements occurs for other cytokine genes, the influence of CHX treatment on cytokine gene expression both before and after stimulation was examined in both EL4 T cells (Figure 4.4), BMDM cells (Figure 4.5) as well as RAW 264.7 macrophages (Figure 4.6). Like the *GM-CSF* gene, *IL-23A* appears to switch from being CHX dependent in EL4 T cells (Figure 4.4) to being a CHX independent gene in macrophages cells (Figures 4.5 & 4.6). *IL-1a*, *Tnfsf9* and *IFN-γ* appear independent of CHX treatment in all cell types (Figures 4.4, 4.5 & 4.6). *IL-6* and *IL-10* in contrast appear to be independent of CHX in EL4 T cells but switch to being inhibited in the presence of CHX in BMDM (Figure 4.5) as well as in RAW 264.7 macrophages (Figure 4.6), although this is less obvious as expression levels are low in this cell line. A summary of CHX dependence based on this data is provided in Table 4.1 along with BRG1 dependence inferred from the literature. From the CHX transcription response data the *IL-6* and *IL-10* genes which are BRG1 independent in T cells were selected for subsequent analysis alongside the *IL-23A* gene and the already characterised *GM-CSF* gene which are BRG1 dependent in T cells. These genes appear to switch in CHX dependence between the two cell types as well as switching in their requirement for the SWI/SNF chromatin remodelling complex. Additionally there is evidence of CHX dependence in both T cells and macrophages for different genes suggesting that CHX dependence is not determined by cell type alone. These genes are therefore ideal candidates to investigate the mechanisms that underlie these changes in gene regulation between cell types.

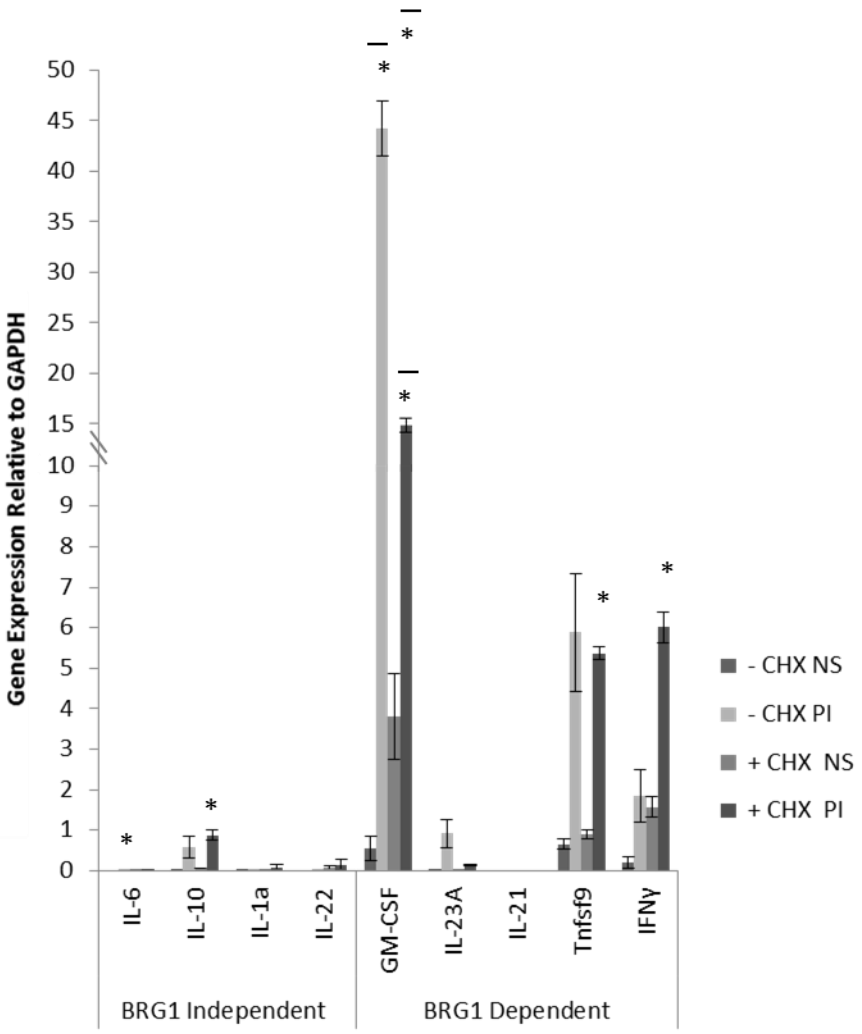

Figure 4.4: Cycloheximide (CHX) dependence of inducible gene activation in EL4 T cells. Murine EL4 T cells were either left untreated or treated with CHX for 30 minutes before incubating with or without PI for 4 hours. In each case, RNA was isolated and cytokine mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown. Significance from NS was determined by Students T. Test * $p<0.05$, ** $p<0.01$.

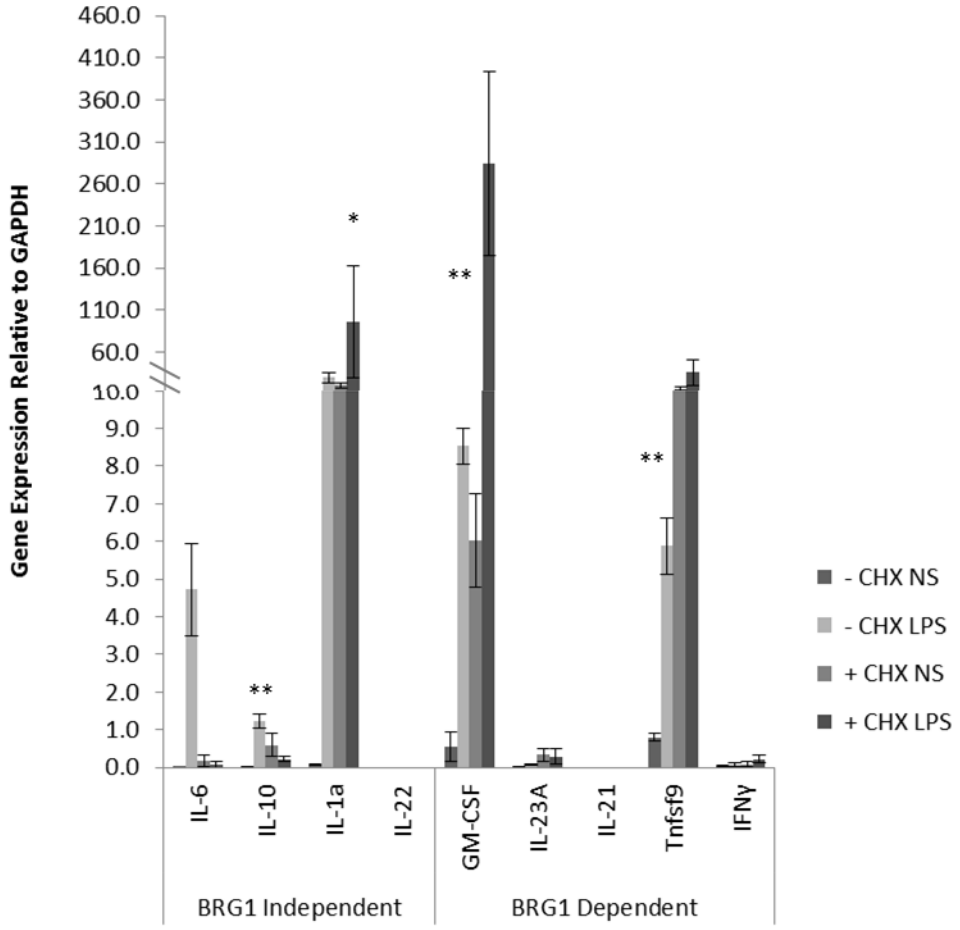

Figure 4.5: Cycloheximide (CHX) dependence of inducible gene activation in bone marrow derived macrophage (BMDM) cells. BMDM cells were either left untreated or treated with CHX for 30 minutes before incubating with or without LPS for 4 hours. RNA was isolated and cytokine mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown. Significance from NS was determined by Students T. Test* $p < 0.05$. ** $p < 0.01$.

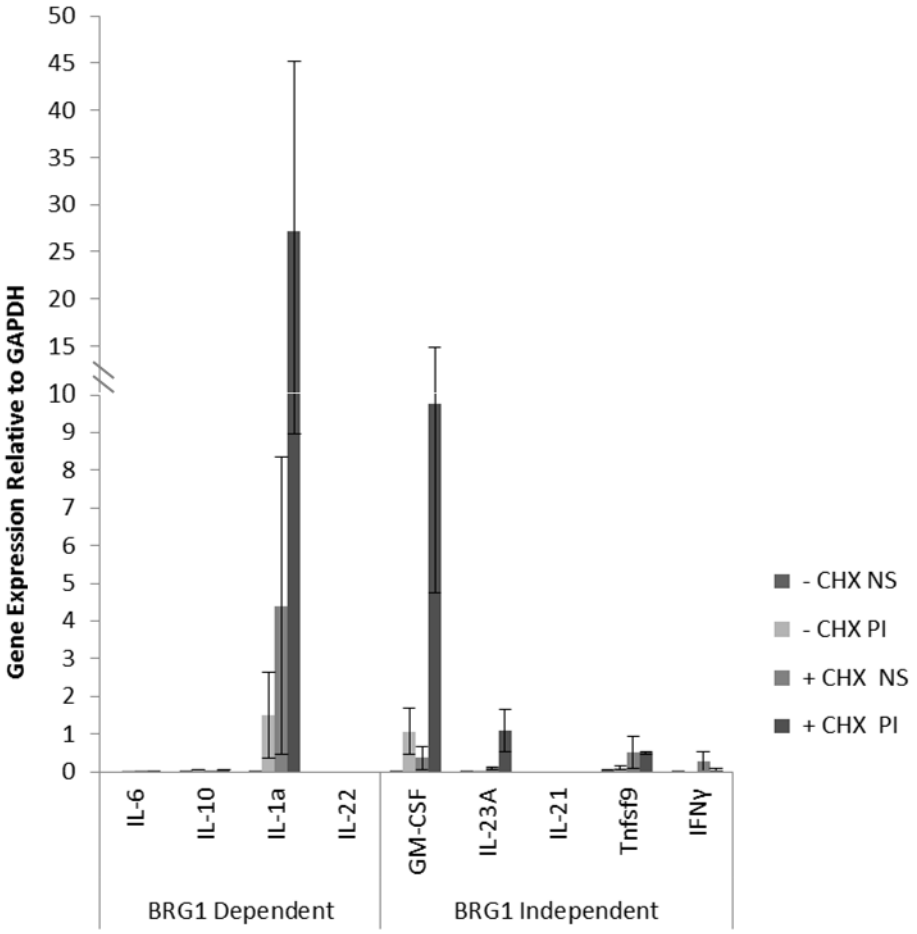

Figure 4.6: Cycloheximide (CHX) dependence of cytokine gene activation in RAW 264.7 macrophage cells. Murine RAW 264.7 macrophage cells were either left untreated or treated with CHX for 30 minutes before incubating with or without LPS for 4 hours. RNA was isolated and cytokine mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown.

Gene	T cells		Macrophages	
	CHX	BRG1	CHX	SWI/SNF
IL-6	Independent	Independent	Dependent	Dependent
IL-10	Independent	Independent	Dependent	Dependent
IL-1a	Independent	Independent	Independent	Independent
IL-22	Independent	Independent	Not expressed	N/A
GM-CSF	Dependent	Dependent	Independent	Independent
IL-23A	Dependent	Dependent	Independent	Independent
IL-21	Not expressed	Dependent	Not expressed	N/A
Tnfsf9	Independent	Dependent	Independent	Independent
IFN-γ	Independent	Dependent	Independent	N/A

Table 4.1: Summary of cycloheximide (CHX) and BRG1 or SWI/SNF dependence of cytokine gene activation in T cells compared to macrophage cells. CHX dependence is determined from the data presented in Figures 4.4-6. Dependence on the BRG1 chromatin remodelling enzyme is based on published data from De, S., A. L. Wurster, et al. (2011). In macrophages reliance on the SWI/SNF family of chromatin remodelling enzymes is based on published data (Ramirez-Carrozzi, V. R., D. Braas, et al. (2009). N/A indicates data was unavailable.

4.2.3 Activation kinetics of inducible genes in T cells compared to macrophages

Previous work has suggested that both the requirement for the synthesis of new proteins and the requirement for chromatin remodelling may be linked to the kinetics of inducible gene activation (Natoli 2009; Natoli et al. 2005; Ramirez-Carrozzi et al. 2009; Saccani, Pantano & Natoli 2001), although this was not found to be the case for *GM-CSF* (Chapter 3). Therefore the kinetics of immune gene activation in response to stimulation were examined in both EL4 T cells and RAW 264.7 macrophages to determine if these differ and whether kinetics reflect the switching in CHX dependence observed for the selected genes between the two cell types. As outlined in the previous chapter for the *GM-CSF* gene, because in some biological replicates basal expression levels were undetectable, in order to effectively compare the kinetics of the response between the two cell types, gene expression relative to *GAPDH* was normalised to expression at 4 hours, which was set to 100 in each case.

In EL4 T cells, *IL-23A* transcript levels increased from 4 hours of PI stimulation. Within the time course examined, the highest levels of mRNA were detected at 24 hours post stimulation (Figure 4.7.A). This is in contrast to the *GM-CSF* gene (Chapter 3) where levels peaked at 8 hours and decline by 24 hours. In RAW 264.7 macrophage cells, an increase in *IL-23A* mRNA levels was observed by 1 hour following LPS stimulation and maintained at this level with a further increase not detected until 24 hours (Figure 4.7.B). These results suggest that the kinetics of *IL-23A* gene activation differ between the two cell types. In T cells, induction follows a similar pattern to that observed for *GM-CSF*, however in macrophage cells the *IL-23A* transcript levels initially increased rapidly and then maintained at a constant level out to 8 hours post stimulation.

IL-6 expression followed a similar upward trend in transcript levels in both EL4 T cells and RAW 264.7 macrophage cells, however there is evidence of a biphasic pattern of

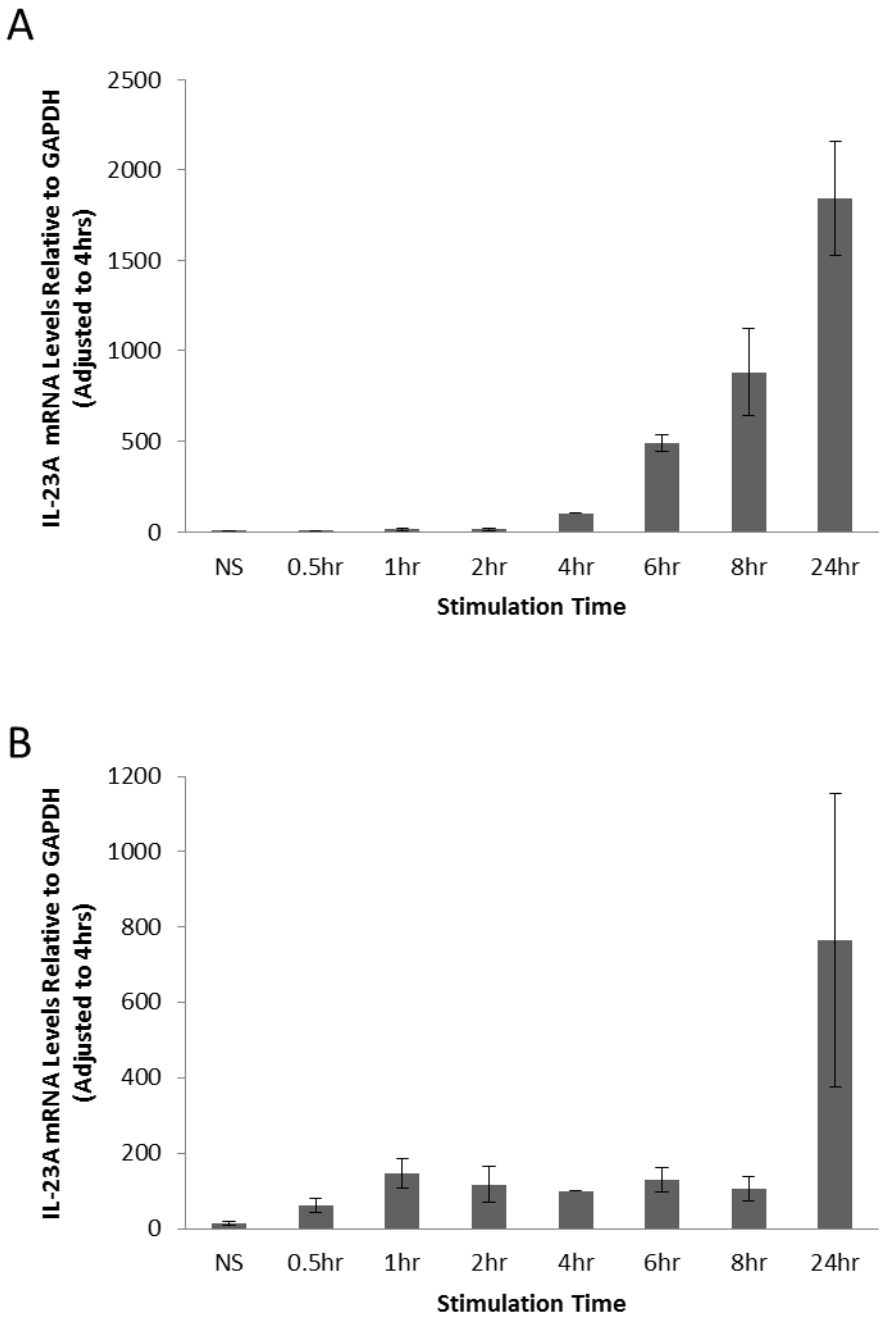

Figure 4.7: Activation kinetics of the IL-23A gene in T cell and macrophage cell lines. (A) Murine EL4 T cells were incubated with PI and (B) Murine RAW 264.7 macrophage cells were incubated with LPS. Cells were either left non-stimulated (NS) or stimulated for the times indicated. In each case, RNA was isolated and IL-23A mRNA levels determined by RT-qPCR and normalised to GAPDH. Gene expression is graphed relative to 4 hours of stimuli. Mean and SEM of at least 3 replicates is shown.

gene transcription. A small initial peak in mRNA levels was observed at 1 hour post stimulation in EL4 T cells and a similar peak detected at 6 hours post stimulation in RAW 264.7 macrophages. These peaks were followed by a decline before maximal mRNA levels were detected at 24 hours (Figure 4.8.A & B). In contrast, *IL-10* expression demonstrated an initial peak at 0.5 hours of stimulation in both T cells and macrophages which decreased at 1 hour before increasing to a greater peak at 6 hours of LPS stimulation and declining again by 24 hours (Figure 4.9.A & B). These results suggest that rather than cell stimulation triggering a cumulative increase in *IL-6* and *IL-10* mRNA, transcription is biphasic. These profiles of activation kinetics are similar in T cells and macrophages indicating that whilst there is a difference in requirement for new protein synthesis between the two cell types, this does not necessarily result in a difference in activation kinetics. This is in keeping with the observations made following examination of the *GM-CSF* gene in the previous chapter, i.e that activation classifications of primary and secondary response genes does not necessarily reflect differences in activation kinetics.

4.2.4 Chromatin remodelling events are associated with increased gene transcription at inducible genes

Changes in chromatin structure at the regulatory region of genes are often required to facilitate subsequent inducible gene expression (Cockerill 2011). To determine if changes in gene expression in T cells and macrophages correlated with changes in chromatin accessibility at the regulatory promoters of the *IL-23A*, *IL-6* and *IL-10* immune genes the CHART qPCR assay was utilised. This technique has previously been shown to be directly comparable between different genes and different cell types, with promoter regions of ubiquitously expressed genes showing comparable levels of MNase1 digestion across different cell lines while developmentally restricted genes demonstrated different accessibility levels (Cruickshank et al. 2008). Promoter primers were selected to amplify the region occupied by the +1 nucleosome which is frequently

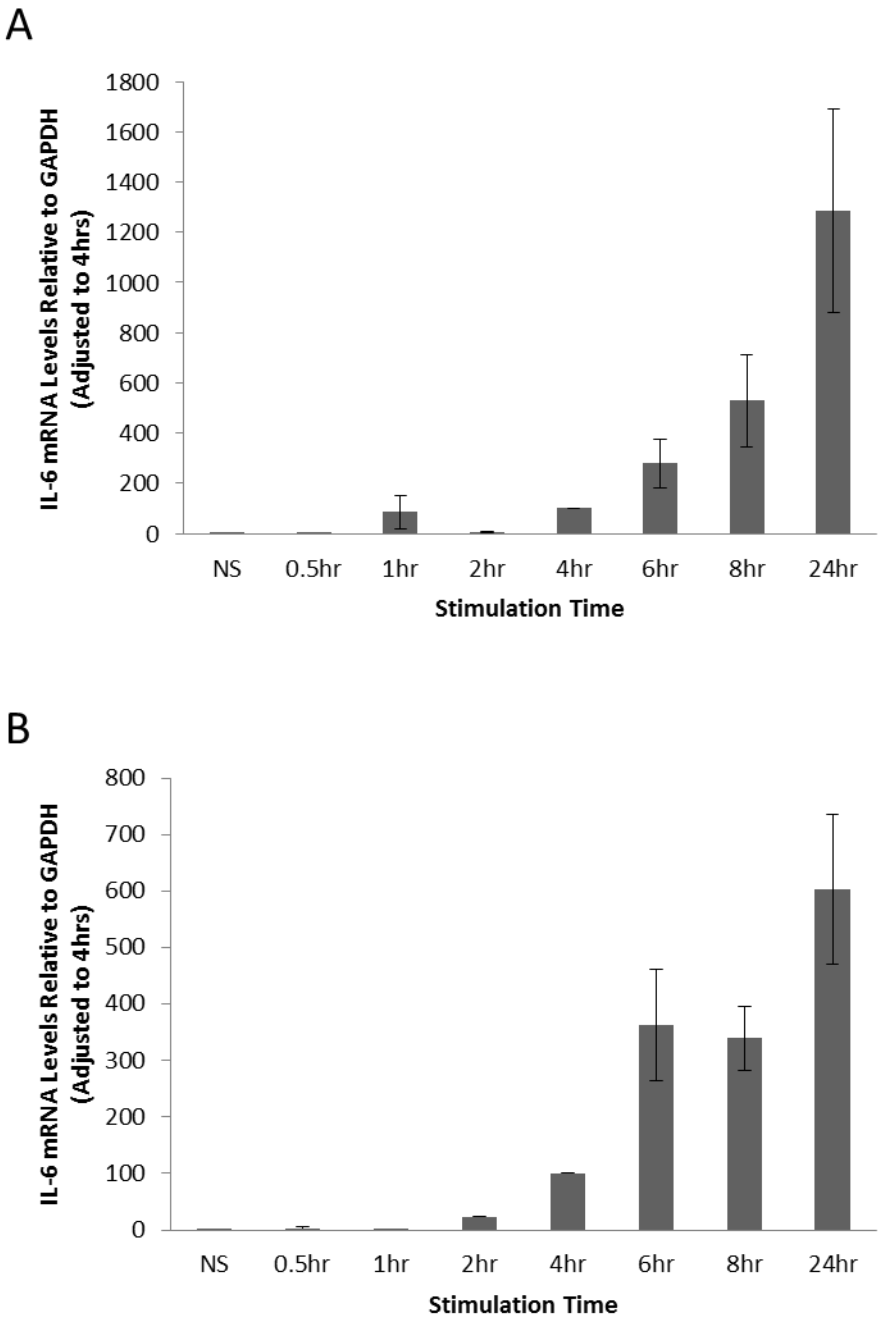

Figure 4.8: Activation kinetics of the IL-6 gene in T cell and macrophage cell lines. (A) Murine EL4 T cells were incubated with PI and (B) Murine RAW 264.7 macrophage cells were incubated with LPS. Cells were either left non-stimulated (NS) or stimulated for the indicated times. In each case, RNA was isolated and IL-6 mRNA levels determined by RT-qPCR and normalised to GAPDH. Gene expression is graphed relative to 4 hours of stimuli. Mean and SEM of at least 3 replicates is shown.

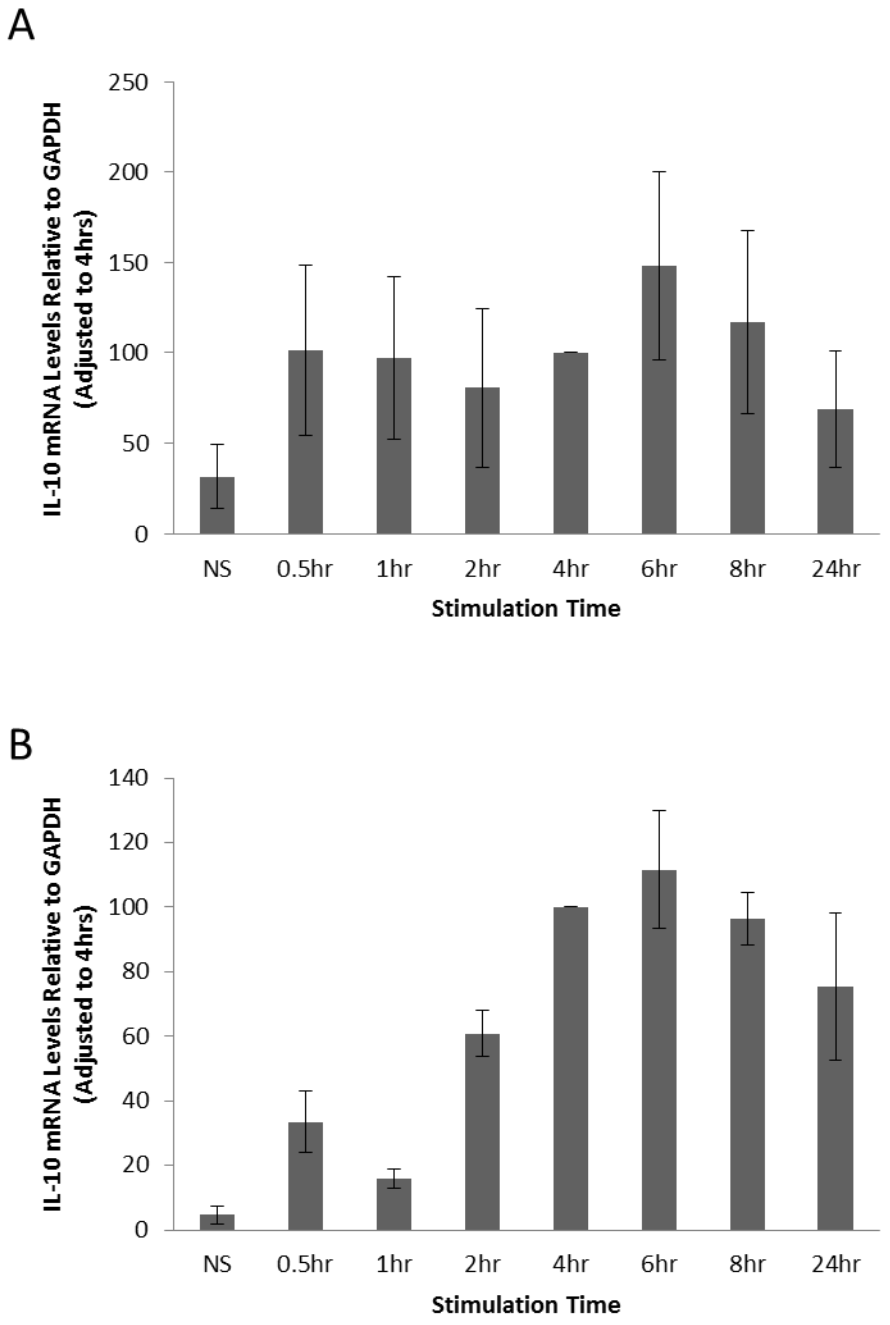

Figure 4.9: Activation kinetics of the IL-10 gene in T cells and macrophage cell lines. (A) murine EL4 T cells were incubated with PI and (B) Murine RAW 264.7 macrophage cells were incubated with LPS. Cells were either left non-stimulated (NS) or stimulated for the times indicated. In each case, RNA was isolated and IL-10 mRNA levels determined by RT-qPCR and normalised to GAPDH. Gene expression is graphed relative to 4hrs of stimuli. Mean and SEM of at least 3 replicates is shown.

removed during transcription (Schones et al. 2008) and have previously been utilised in the literature (refer to Table 2.4, Materials and Methods).

In EL4 T cells, when the promoter regions of *IL-23A*, *IL-6* and *IL-10* were examined using the chromatin accessibility assay, accessibility of less than one was detected (Figure 4.10.A) which indicates that the non-digested samples contain less intact genomic DNA than the samples subjected to MNase1. As discussed in Chapter 3 this may be attributed to inaccuracy in the DNA quantitation and is not considered biologically significant (Liang et al. 2006). Prior to stimulation similar levels of basal promoter accessibility are seen for all genes examined. Stimulation for 4 hours with PI results in an increase in accessibility at both the *IL-23A* ($p= 0.14$) and *IL-6* ($p= 0.38$) promoter region in EL4 T cells, although these increases were not statistically significant, and nor of the magnitude of that observed at the *GM-CSF* promoter (Figure 4.10). In contrast the *IL-10* promoter showed no increase in accessibility in response to stimulation. The increase in the *IL-23A*, *IL-10* and *IL-6* mRNA levels is not as large as that observed for the *GM-CSF* gene (Figure 4.4), conceivably this is because stimulation only induces low level transcription which is permissible without significant chromatin remodelling.

In RAW 264.7 macrophage cells, both the *IL-6* and *IL-10* promoters appear to have relatively accessible basal chromatin structure in comparison to the *GM-CSF* and *IL-23A* promoter regions (Figure 4.10.B), with basal accessibility greater than 1. An increase in promoter accessibility is then seen in response to LPS stimulation at all promoter regions suggesting that chromatin remodelling occurs in response to stimulation enabling increased gene transcription to occur. However, this change in accessibility is not statistically significant for the *IL-6* promoter ($p= 0.17$). Again the changes observed are not of the magnitude of that seen at the *GM-CSF* promoter in T cells.

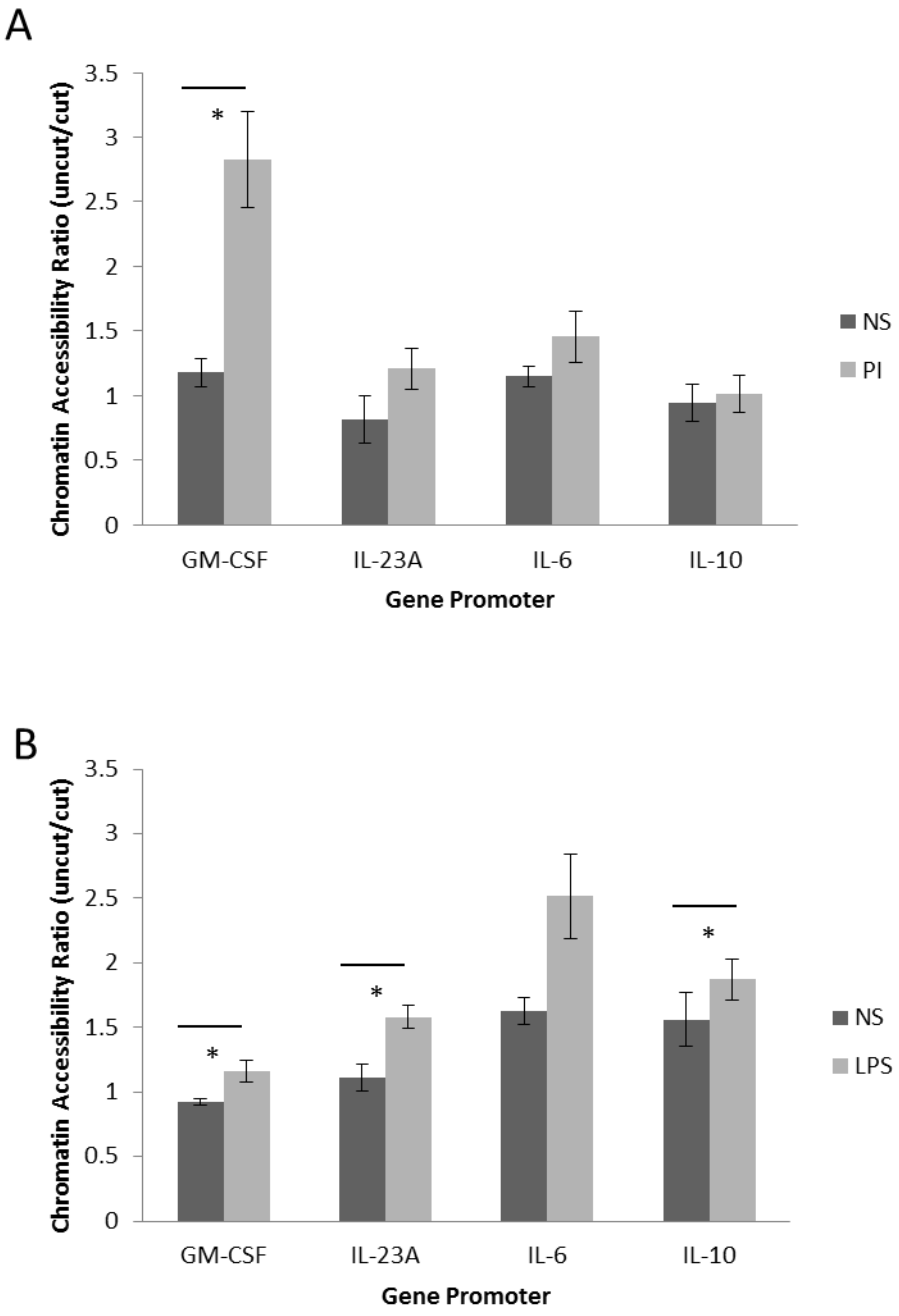

Figure 4.10: Promoter accessibility in macrophages compared to T cells. (A) Murine EL4 T cells were either left non-stimulated (NS) or stimulated with PI for 4 hours. (B) Murine RAW 264.7 macrophages were either left NS or stimulated with LPS for 4 hours. Following stimulation, nuclei were prepared and equal portions were either left non-digested or digested with MNase1. In each case, genomic DNA was isolated and analysed by qPCR. The ratio of undigested to digested PCR product was determined (uncut/cut). Mean and SEM of at least 3 replicates is shown. Significance from NS was determined by Students T. Test * $p<0.05$.

These results show that low levels of gene transcription are permissible with only small increases in chromatin accessibility. The level of chromatin remodelling taking place at the promoter region is not as large for *IL-23A*, *IL-6* and *IL-10* as is observed at the *GM-CSF* promoter in T cells, which suggest that the extent of chromatin remodelling may dictate the magnitude of the mRNA transcription response. In macrophage cells the *IL-6* and *IL-10* promoter regions are held in more accessible basal state than the *GM-CSF* or *IL-23A* promoters demonstrating that there is some variation in the inherent level of basal accessibility which may impact on the level of chromatin remodelling required for increased gene transcription.

4.2.5 Regulation of cytokine gene responses by c-Rel

In Chapter 3 it was demonstrated that nuclear localisation of the c-Rel transcription factor was inhibited in CHX treated T cells but not macrophages suggesting that c-Rel regulation may partially explain the divergence in gene expression responses between the two cell types. Genome-wide data was used to identify potential c-Rel dependent immune genes in T cells which were selected for this study. Therefore to confirm c-Rel dependence in both T cells and macrophages the c-Rel inhibitor pentoxifylline (PTX) was employed to examine the effect of c-Rel inhibition on inducible gene expression. *IL-23A* mRNA levels increased in response to PI treatment of EL4 T cells but this response was diminished in the presence of PTX (Figure 4.11.A; $p= 0.178$). Likewise in RAW 264.7 cells, a significant increase in *IL-23A* mRNA levels is observed in response to LPS treatment (Figure 4.11.B; $p<0.05$) but this response is inhibited in the presence of PTX (Figure 4.11.B; $p= 0.270$). These results indicate that *IL-23A* gene activation is reliant on c-Rel in both T cells and macrophages.

IL-6 mRNA levels are relatively low in both un-stimulated T cells (Figure 4.12.A) and macrophages (Figure 4.12.B) both in the presence and absence of PTX. An increase in

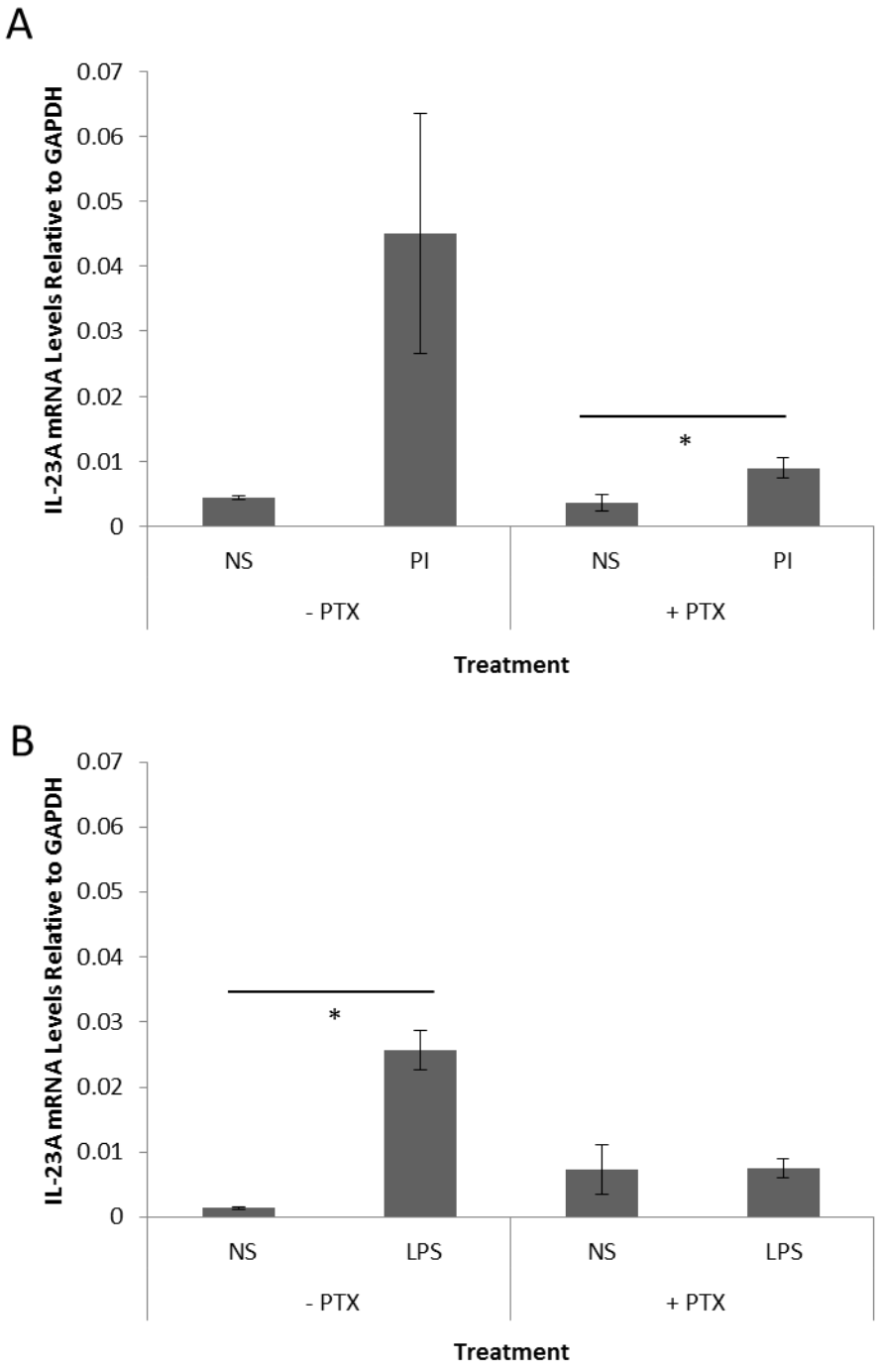

Figure 4.11: Pentoxifylline (PTX) inhibition of IL-23A gene activation in T cells and macrophages. (A) Murine EL4 T cells were either left untreated or treated with PTX for 30 minutes before incubating with or without PI for 4 hours. (B) Murine RAW 264.7 macrophage cells were either left untreated or treated with PTX for 30 minutes before incubating with or without LPS for 4 hours. In each case, RNA was isolated and IL-23A mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown. The significance from NS was determined by Students T. Test as was the significance of the change in gene expression between –PTX and +PTX treatments, * $p<0.05$.

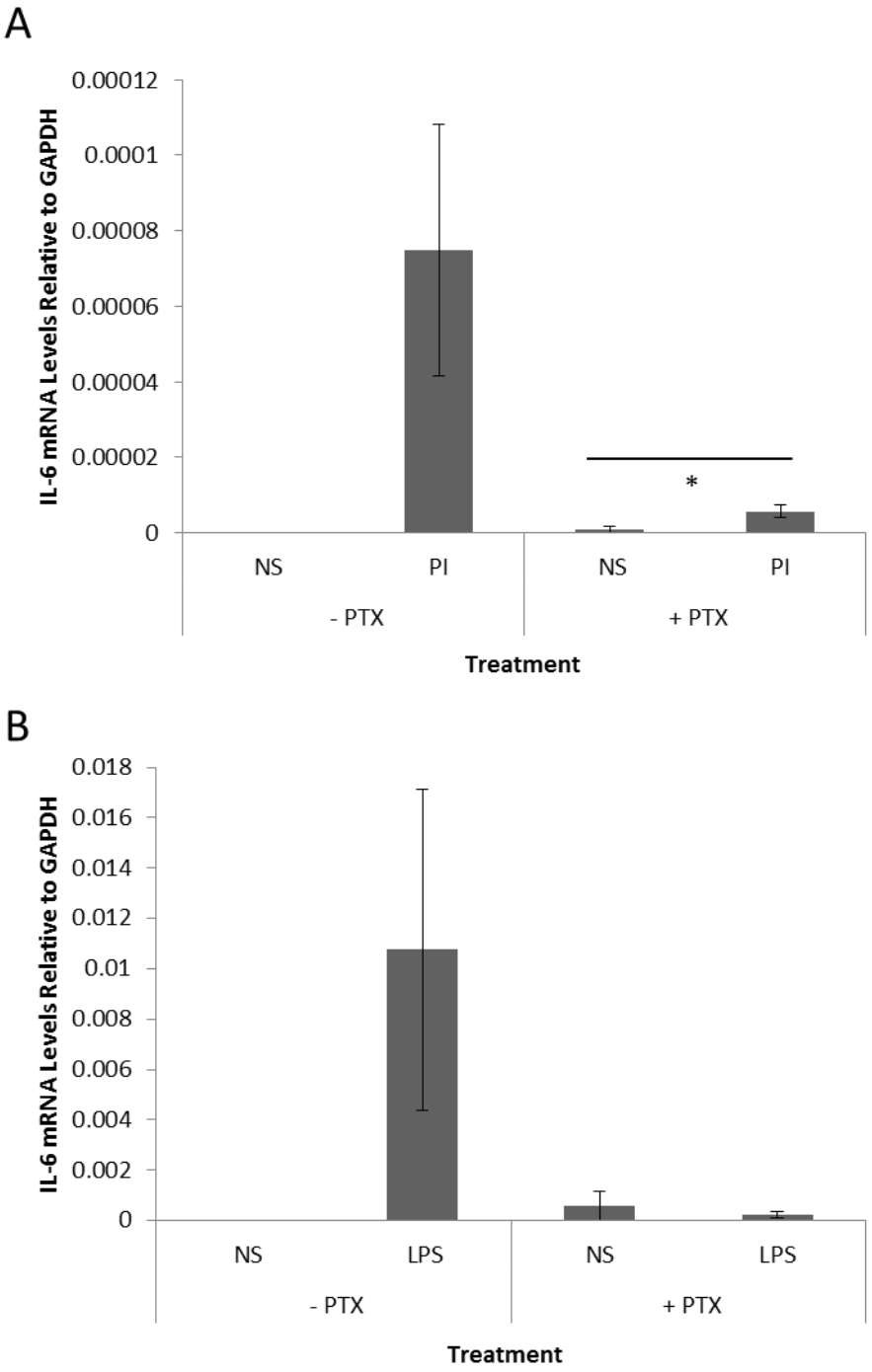

Figure 4.12: Pentoxifylline (PTX) inhibition of IL-6 gene activation in T cells and macrophages. (A) Murine EL4 T cells were either left untreated or treated with PTX for 30 minutes before incubating with or without PI for 4 hours. (B) Murine RAW 264.7 macrophage cells were either left untreated or treated with PTX for 30 minutes before incubating with or without LPS for 4 hours. In each case, RNA was isolated and IL-6 mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown. The significance from NS was determined by Students T. Test as was the significance of the change in gene expression between –PTX and +PTX treatments, * $p<0.05$.

IL-6 mRNA levels is observed in response to stimulation and is inhibited by the presence of PTX in both cell types, suggesting that c-Rel is also required for transcriptional activation of *IL-6*. Whilst there is no significant increase in *IL-10* mRNA levels in response to PI treatment of T cells, PTX treatment results in a decrease in basal mRNA levels (Figure 4.13.A) suggesting that c-Rel is required for basal transcription of *IL-10* gene expression in T cells. In RAW 264.7 macrophage cells, there is a significant increase in *IL-10* mRNA levels in response to stimulation (Figure 4.13.B; $p<0.05$) and this response is slightly reduced in the presence of PTX, however this is not significant.

Thus c-Rel inhibition resulted in reduced inducible expression of *IL-23A*, *IL-6* and *IL-10* in both T cells and macrophages suggesting that, as predicted by the genome-wide studies, the c-Rel transcription factor is required for activation of these genes. Furthermore, basal IL-10 expression was reduced in the presence of PTX suggesting that c-Rel is also required to maintain its basal levels of gene transcription.

Pentoxifylline is a pharmacological inhibitor of c-Rel which is used clinically as a vasoactive anti-inflammatory drug which clearly acts on a diverse range of cell types. However little is known about the other signalling pathways which may be affected by PTX (Wang et al. 1997). In order to examine the role of c-Rel in inducible gene expression independent of any possible off target effects of PTX treatment, gene expression responses were examined using cells derived from c-Rel$^{-/-}$ mice compared to C57/BL6 (WT) mice. CD4+ T cells were isolated from mice and either left un-stimulated or stimulated with PI and CD28 for 4 hours. BMDM cells were differentiated from bone marrow and either left un-stimulated or stimulated for 4 hours with LPS. Following stimulation RNA was extracted and examined by RT-qPCR. In comparison to wild type cells, both CD4+ T cells and BMDM derived from c-Rel$^{-/-}$ mice exhibited low induction of *IL-23A* mRNA in response to stimulation (Figure 4.14.A & B). *IL-6* mRNA levels increased upon stimulation in WT CD4+ T cells and BMDM cells as expected. However, this response was greater in both cell types derived

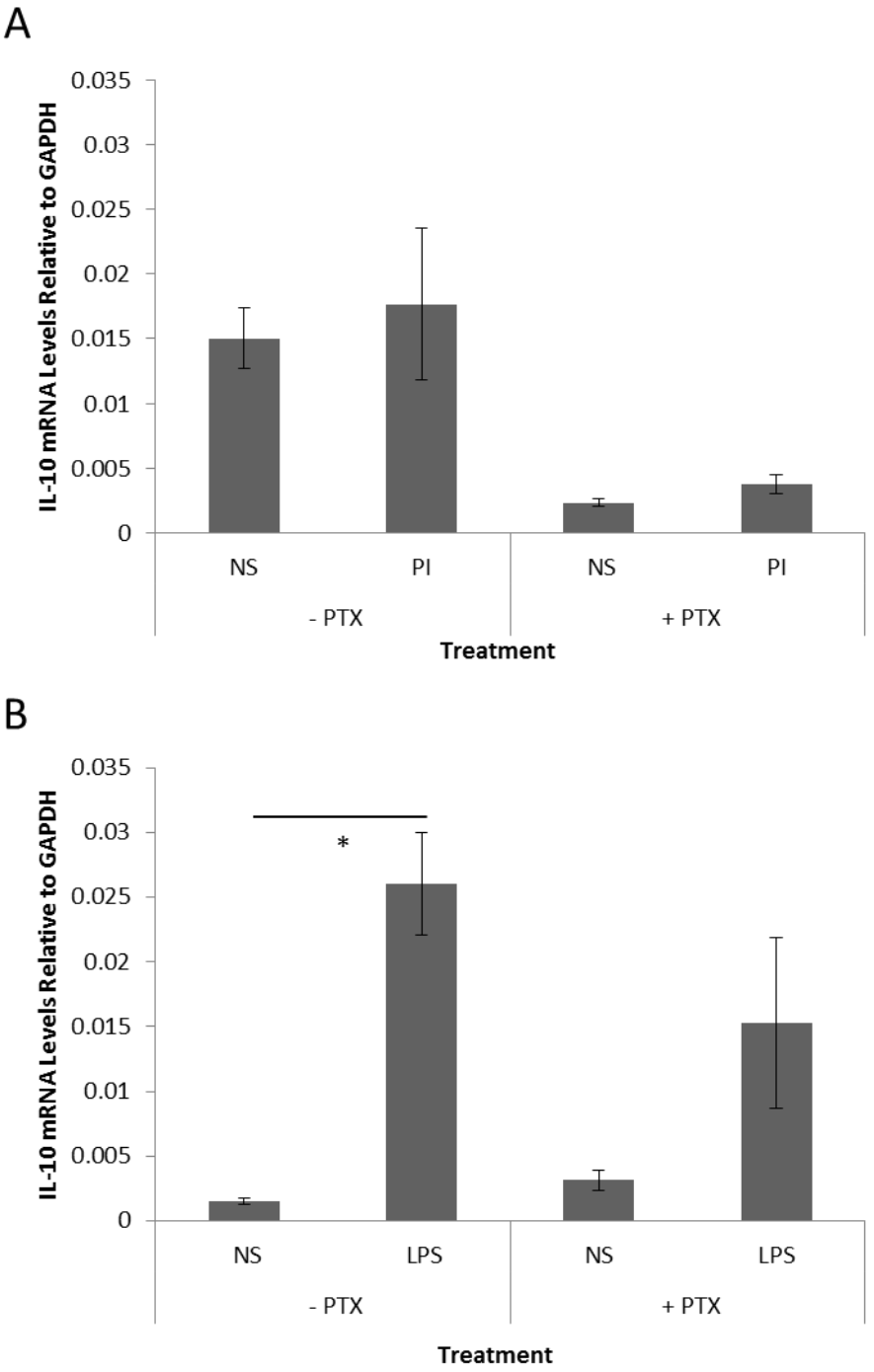

Figure 4.13: Pentoxifylline (PTX) inhibition of IL-10 gene activation in T cells and macrophages. (A) Murine EL4 T cells were either left untreated or treated with PTX for 30 minutes before incubating with or without PI for 4 hours. (B) Murine RAW 264.7 macrophage cells were either left untreated or treated with PTX for 30 minutes before incubating with or without LPS for 4 hours. In each case, RNA was isolated and IL-10 mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown. The significance from NS was determined by Students T. Test as was the significance of the change in gene expression between –PTX and +PTX treatments, * $p<0.05$.

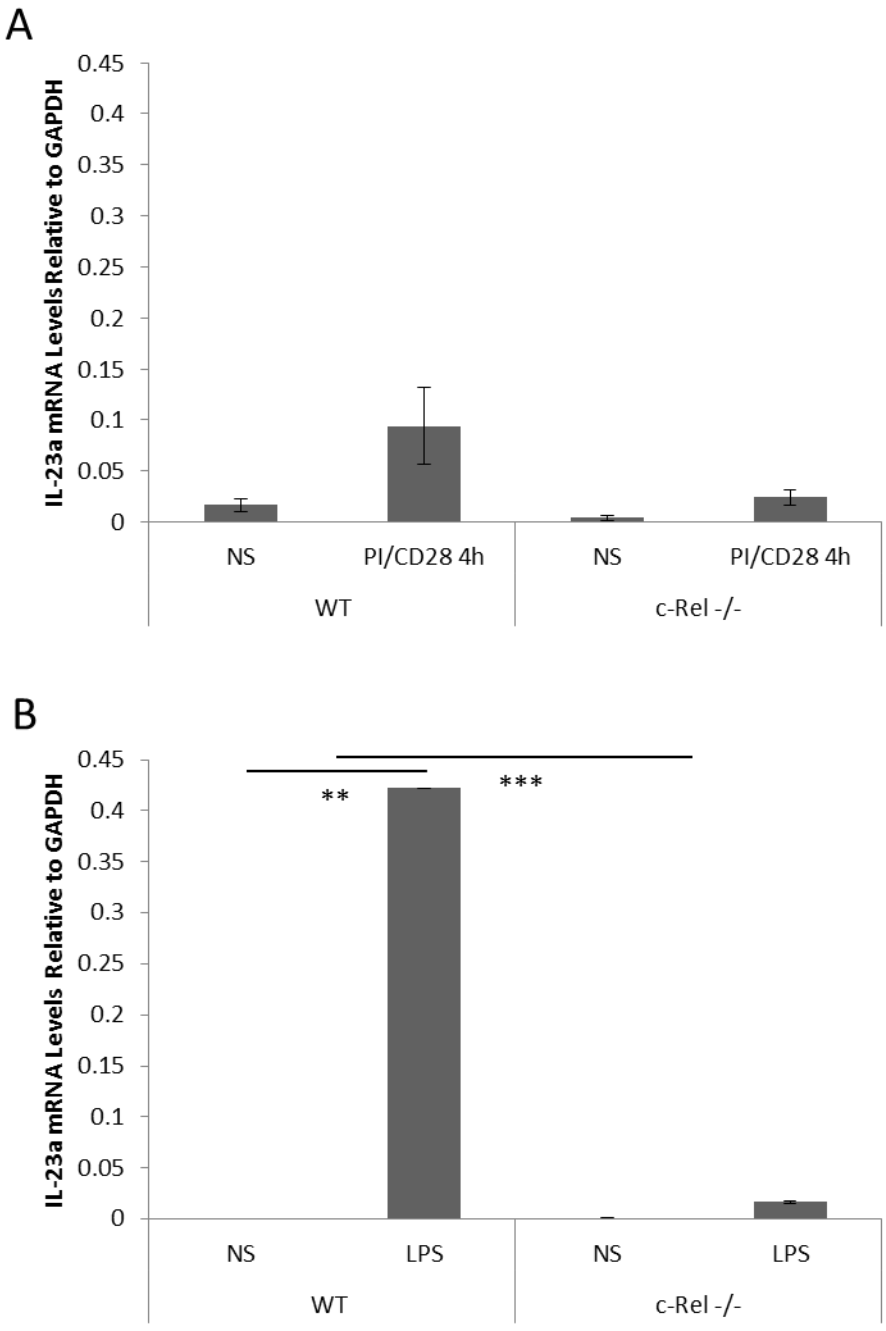

Figure 4.14: IL-23A gene activation reliance on c-Rel; (A) CD4+ T cells were isolated from wild type C57/BL6 (WT) and c-Rel$^{-/-}$ mice. Cells were either non-stimulated (NS) or stimulated with PMA and calcium ionophore in conjunction with CD28 for 4 hours (PI/CD28). (B) Bone marrow derived macrophages were differentiated from WT and c-Rel$^{-/-}$ mice. Cells were either NS or stimulated with LPS for 4 hours. In each case, RNA was isolated and IL-23A mRNA levels detected by RT-qPCR and adjusted to GAPDH. Mean and SEM of at least 3 replicates is shown. Significance from NS was determined by Students T. Test as was the significance of the change from NS between WT and c-Rel$^{-/-}$ cells, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

from c-Rel$^{-/-}$ cells (Figure 4.15.A & B). Similarly, while *IL-10* mRNA increased in response to stimulation in WT cells, in c-Rel$^{-/-}$ cells the *IL-10* mRNA response was also enhanced with a significant difference in fold change observed for *IL-10* in BMDM (Figure 4.16.A & B. p= <0.05).

These data suggest that while c-Rel is required for activation of some genes, including *GM-CSF* (Chapter 3) and *IL-23A*, c-Rel has a negative regulatory role for *IL-6* and *IL-10*. Furthermore, the increased gene expression response in the absence of c-Rel is in contrast to what was observed for *IL-6* and *IL-10* mRNA levels in the presence of PTX, suggesting that the PTX effect on gene transcription may be due to the non-specific effects on factors other than c-Rel.

4.2.6 Association of NF-κB transcription factors with immune genes

With the intention of discerning if the role of c-Rel in cytokine gene regulation was attributable to direct interaction between the transcription factor and the promoter regions or through indirect mechanisms, chromatin immunoprecipitation (ChIP) was utilised. Association of the NF-κB transcription factors, Rel-A and c-Rel with the promoter regions of the immune cytokine genes both before and after stimulation in T cells was examined. As previously documented an increase in the association between both c-Rel (Figure 4.17.A) and Rel-A (Figure 4.17.B) is detectible at the *GM-CSF* promoter region upon PI stimulation (Poke et al. 2012). Both c-Rel and Rel-A also showed an increased association with the *IL-23A* promoter (Figure 4.17.A & B). In addition, increased association of Rel-A and c-Rel with the *IL-6* promoter was observed upon stimulation, although no association was seen with the *IL-10* promoter (Figure 4.17.A & B). The levels of the two NF-κB proteins associated with the *IL-10* promoter are comparable to that of the inactive Rhodopsin control region which is not expressed in haematopoietic cells and is not regulated by c-Rel. These data suggest that *IL-10* may

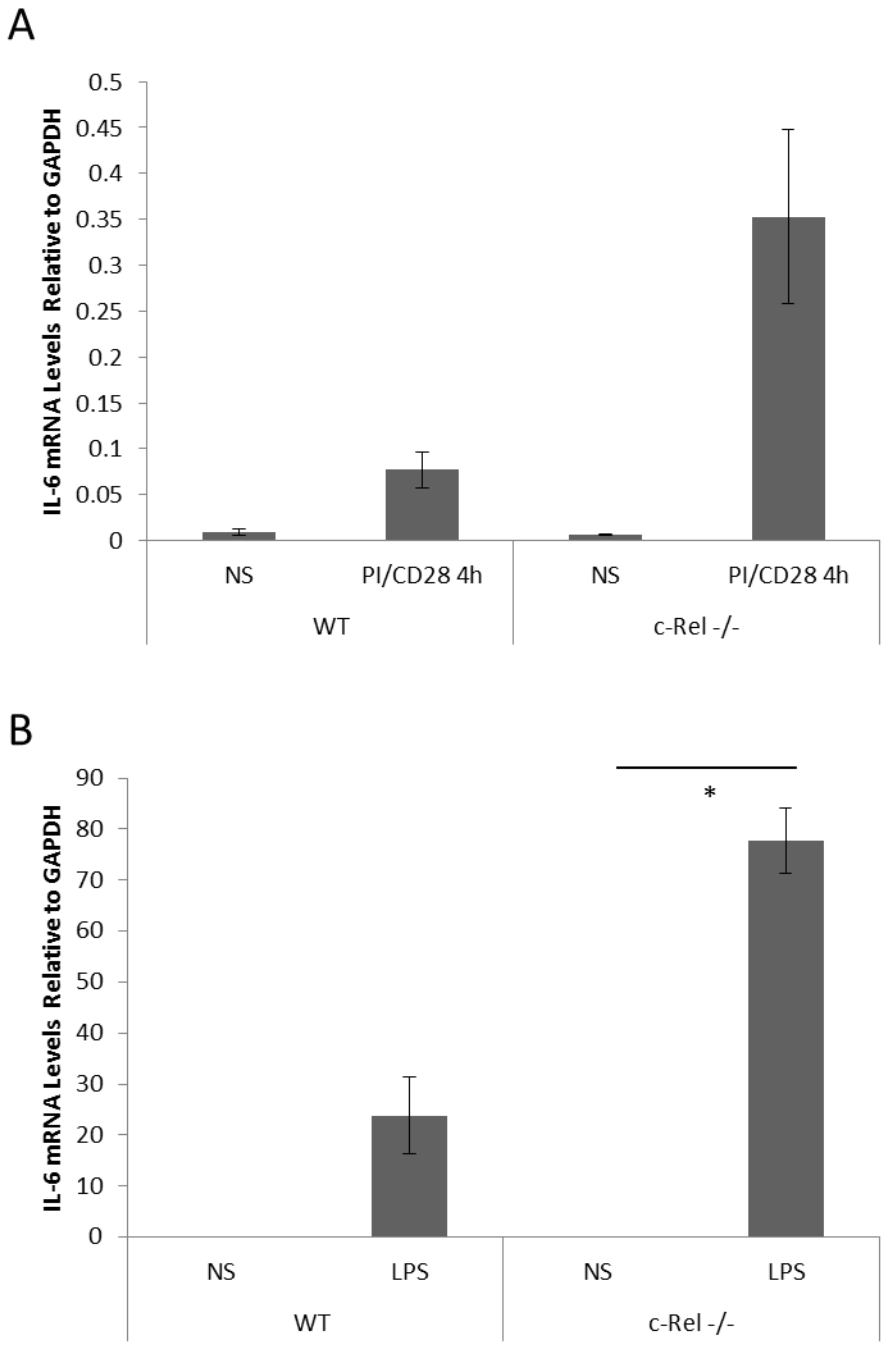

Figure 4.15: IL-6 gene activation reliance on c-Rel; (A) CD4+ T cells were isolated from wild type C57/BL6 (WT) and c-Rel$^{-/-}$ mice. Cells were either non-stimulated (NS) or stimulated with PMA and calcium ionophore in conjunction with CD28 for 4 hours (PI/CD28). (B) Bone marrow derived macrophages were differentiated from WT and c-Rel$^{-/-}$ mice. Cells were either NS or stimulated with LPS for 4 hours. In each case, RNA was isolated and IL-6 mRNA levels detected by RT-qPCR and adjusted to GAPDH. Mean and SEM of at least 3 replicates is shown. Significance from NS was determined by Students T. Test as was the significance of the change from NS between WT and c-Rel$^{-/-}$ cells, * $p<0.05$.

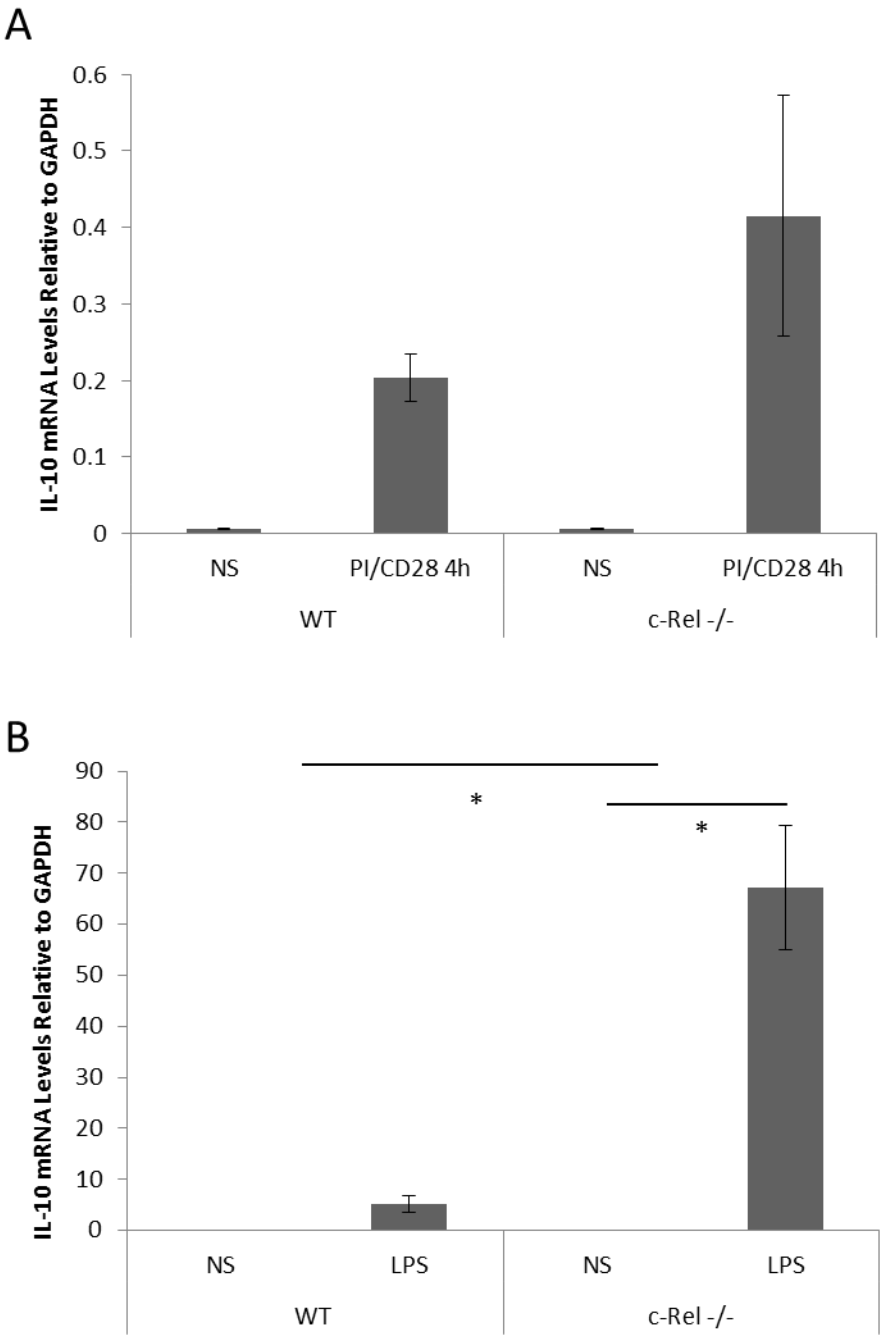

Figure 4.16: IL-10 gene activation reliance on c-Rel; (A) CD4+ T cells were isolated from wild type C57/BL6 (WT) and c-Rel$^{-/-}$ mice. Cells were either non-stimulated (NS) or stimulated with PMA and calcium ionophore in conjunction with CD28 for 4 hours (PI/CD28). (B) Bone marrow derived macrophages were differentiated from WT and c-Rel $^{-/-}$ mice. Cells were either NS or stimulated with LPS for 4 hours. In each case, RNA was isolated and IL-10 mRNA levels detected by RT-qPCR and adjusted to GAPDH. Mean and SEM of at least 3 replicates is shown. Significance from NS was determined by Students T. Test as was the significance of the change from NS between WT and c-Rel$^{-/-}$ cells, * $p<0.05$.

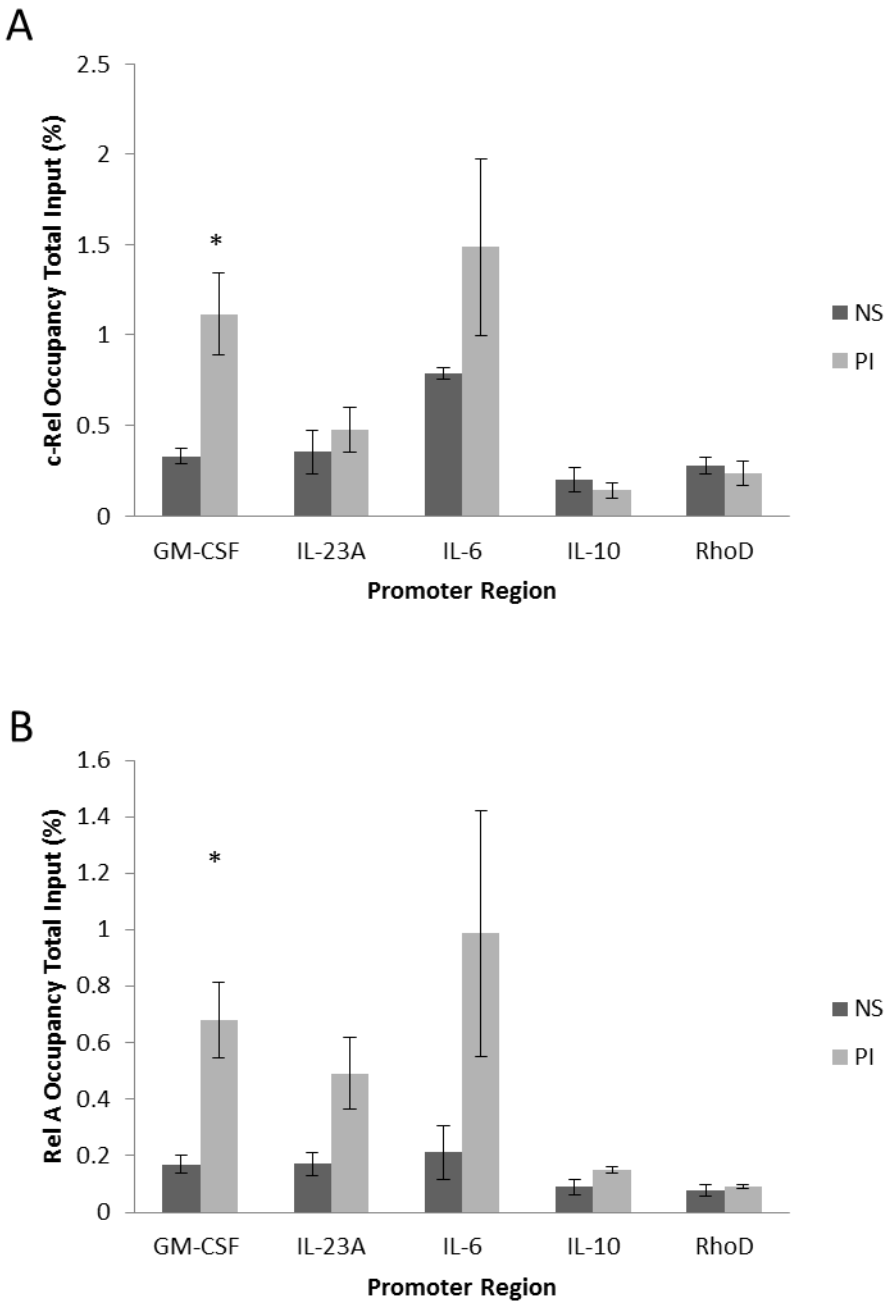

Figure 4.17: Association of NF-κB transcription factors with cytokine gene promoters in T cells. Chromatin immunoprecipitation (ChIP) for c-Rel (A) and Rel A (B) was performed in non-stimulated (NS) and stimulated (PI) EL4 T cells. Association with the gene promoters was determined by qPCR and normalised to the inactive rhodopsin promoter region. Mean and SEM of at least 3 replicates is shown. Significance from NS was determined by Students T. Test * $p<0.05$.

not be regulated by direct c-Rel or Rel-A binding at the promoter region. Instead, *IL-10* could be regulated by c-Rel binding to a more distal location or via an indirect mechanism. To determine which of these is likely to be the case the NF-κB binding sites were determined bioinformatically and schematically depicted relative to the proximal promoter region amplified by the promoter primers (Figure 4.18). The *GM-CSF*, *IL-23A* and *IL-6* promoter regions amplified by the primer sets selected overlap with the putative NF-κB binding sites. In contrast the region amplified by the *IL-10* promoter primers is approximately 500bp from the region amplified. Binding of c-Rel at this site may be undetectable due to the level of DNA shearing employed in the ChIP protocol. In order to examine the association between c-Rel and the upstream NF-κB binding site a new primer set would need to be designed specific for this region.

These results suggest that *GM-CSF*, *IL-23A* and *IL-6* are regulated by c-Rel through direct association of c-Rel with their respective promoter regions. Gene expression analysis in c-Rel$^{-/-}$ cells indicates that c-Rel has a positive influence on *IL-23A* expression and negative regulatory role in *IL-6* expression. Whilst gene expression analysis of *IL-10* also suggests that the gene is negatively regulated by c-Rel, no association between the promoter region and c-Rel was observed. Bioinformatic analysis suggests that the *IL-10* NF-κB binding site is located upstream of the proximal promoter region, and binding at this site may therefore confer gene repression. These results suggest that c-Rel may be both a positive and negative regulator of immune gene expression.

4.2.7 NF-κB transcription factors driving promoter activity

To examine how both positive and negative changes in gene expression were driven by c-Rel binding at the gene promoter regions of the selected genes a luciferase assay was performed. DNA fragments were amplified that encompassed the *IL-23A*, *IL-6* and *IL-10 5*' regions (approximately 1000bp in length) and each was ligated into the pXPG

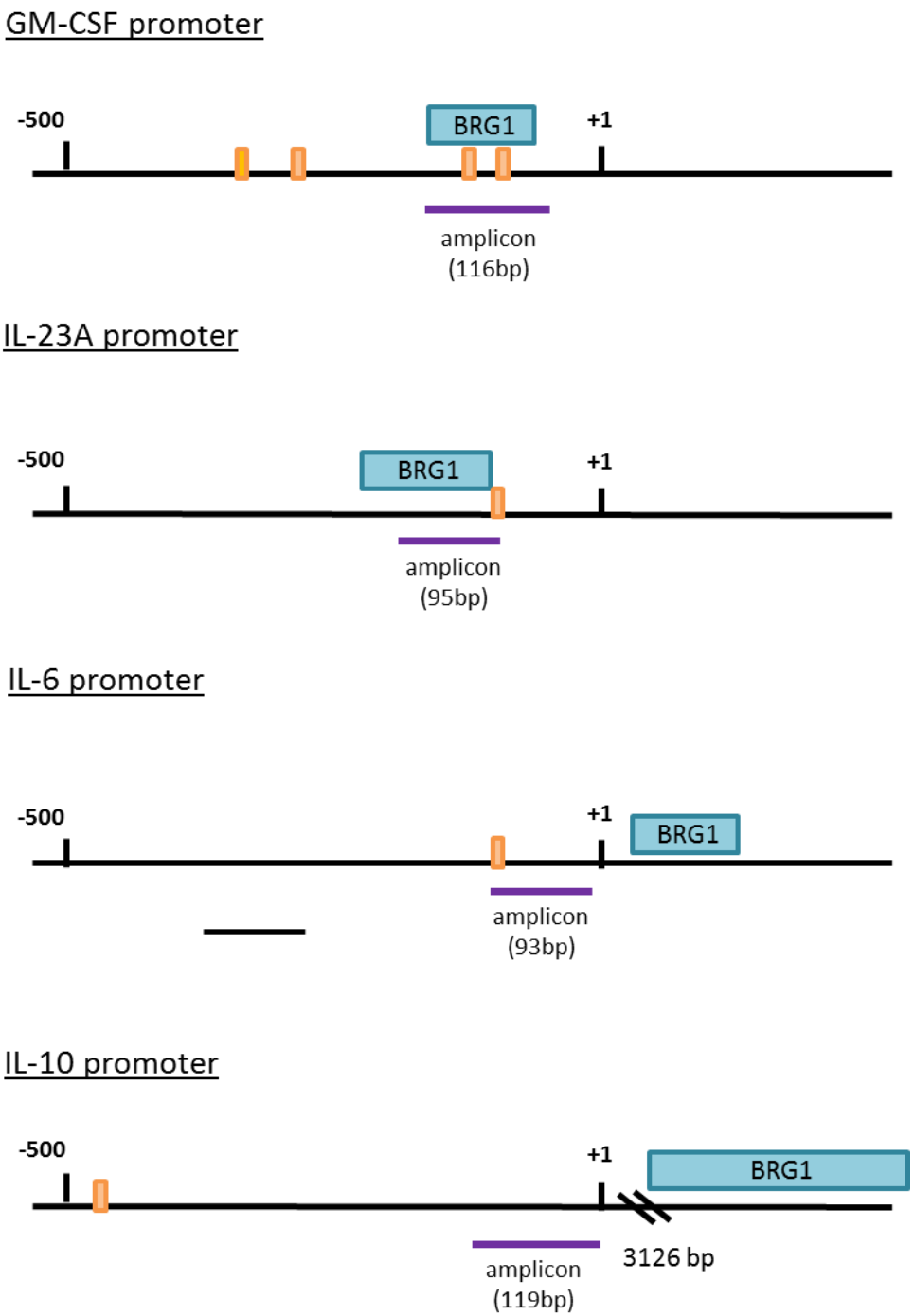

Figure 4.18: Schematic representation of BRG1 and NF-κB binding sites across the cytokine gene promoters relative to the +1 transcriptional start site. Putative BRG1 binding sites are shown in blue, NF- κB binding sites are indicated in orange. The location of the promoter fragment amplified by the promoter primer sets used in the ChIP and CHART assays is indicated in purple. In the case of the IL-10 the putative BRG1 binding site is located 3126 bp upstream of the +1 transcription start site.

reporter vector. The pXPG vector contains the luciferase gene and allows regulation of the luciferase gene by the cloned promoters (Bert et al. 2000). These promoter reporter constructs were transfected into EL4 T cells and RAW 264.7 macrophage cells along with c-Rel (cFlag pcDNA3) and Rel-A (cFlag pcDNA3) expression plasmids (Sanjabi et al. 2005). Each promoter pXPG reporter plasmid was individually co-transfected with Rel-A, c-Rel or a CMV control. The relative luciferase activity driven by Rel-A or c-Rel is shown normalised to the activity detected for the cells transfected with the CMV control. In transfected EL4 T cells, Rel-A drives an increase in *IL-23A*, *IL-6* and *IL-10* promoter activity relative to the CMV control (Figure 4.19.A). c-Rel also increased promoter activity above that of the CMV control for all promoter regions. However the magnitude of this increase is not as substantial as that driven by Rel-A (Figure 4.19.A).

In RAW 264.7 macrophage cells, transfection of the *IL-23A* promoter with Rel-A resulted in an increase in promoter activity compared to the CMV control (Figure 4.19.B). In contrast to what was observed in the EL4 T cells, promoter activity was more substantial when transfected with c-Rel (Figure 4.19.B). *IL-6* promoter activity was decreased below that of the CMV control when co-transfected with Rel-A (Figure 4.19.B; $p = <0.05$). c-Rel also had a negative effect on *IL-6* promoter activity however this was not significantly less than the control (Figure 4.19.B). The *IL-10* promoter activity is similar when transfected into RAW 264.7 cells to what was observed in EL4 cells with an increase in activity driven by both Rel-A and c-Rel, but with the Rel-A effect being more substantial (Figure 4.19.B). Each of these transfections was performed as 3 individual biological replicates on separate days. Viewed individually the trends in promoter activity are the same, however the magnitude of the luciferase activity detected varied between each experiment. This variation means that the changes in activity are not statistically significant except in the case of the *IL-6* promoter and Rel-A repression in macrophages (Figure 4.19.B).

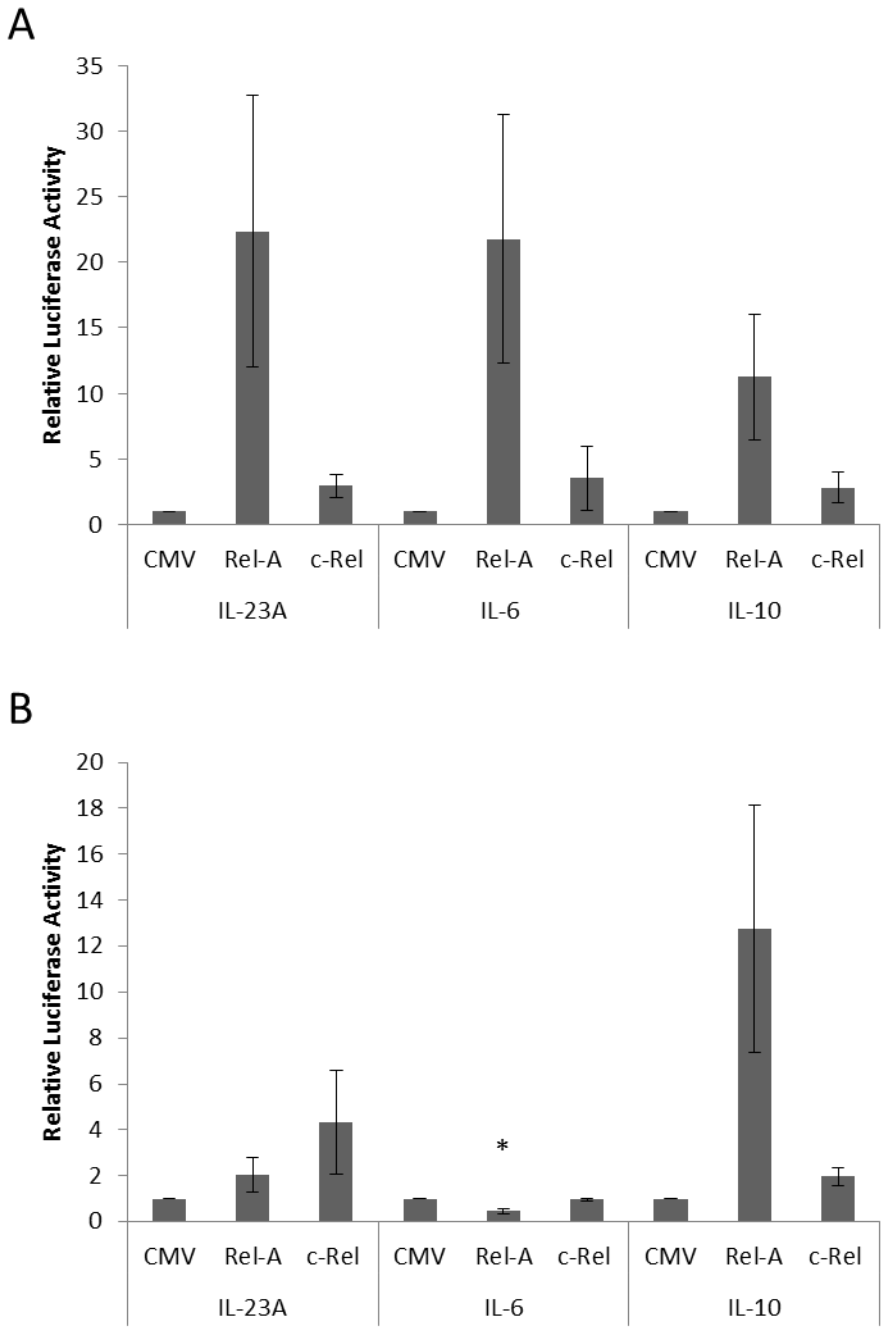

Figure 4.19: Promoter activity in the presence of Rel-A and c-Rel. Luciferase assays were performed in EL4 T cells (A) and RAW264.7 macrophages (B). Cells were transfected with PXPG reporter construct ligated with IL-23A, IL-6 or IL-10 promoter region and either the CMV control plasmid, Rel-A or c-Rel. 24hrs post transfection the cells were lysed and 30µg of cell lyses was combined with luciferase reagent and activity determined by luminometer. Data is normalised to CMV activity. Mean and SEM of at least 3 replicates is shown. Significance from CMV control was determined by Students T. Test. *$p<0.05$.

The luciferase assay analysis confirms that both the Rel-A and c-Rel transcription factors are able to increase transcription driven by the *IL-23A* promoter fragment. In contrast to what was observed in the c-Rel knockout cells, transfection with either Rel-A or c-Rel increased transcription driven by the *IL-10* promoter fragment in both cell types. IL-6 promoter activity switched between T cells and macrophages. In EL4 T cells either Rel-A or c-Rel transfection increased transcription from the IL-6 promoter. The same transfections in macrophage cells resulted in decreased transcription from the IL-6 promoter. These results provide further evidence that c-Rel may act as both a positive and negative regulator of gene expression.

4.3 Discussion

Analysis of genome-wide data revealed that a large cohort of genes is potentially regulated by the co-ordinated activity of the transcription factor c-Rel and the chromatin remodelling complex BRG1. Although identified from data produced in CD4+ T cells, the majority of cytokine genes selected for further study were also induced in response to LPS in macrophage cells, with the highest level of gene expression observed for *GM-CSF* and *IL-1a*, both of which have previously been reported to be strong responders to LPS stimulation (Iglesias et al. 2012; Li et al. 2012).

4.3.1 Selection of genes for further analysis

The work in this chapter explores the mechanisms of "switching" in CHX dependence as this may be a common mechanism by which appropriate expression of immune genes is maintained. From the larger group of c-Rel dependent genes *IL-23A* was selected for more detailed analysis as it showed evidence of switching from a secondary response gene in T cells to a primary response gene in macrophages. IL-6 and IL-10 were selected as they switch in the opposite direction, from primary response genes in T cells to secondary responders in macrophages.

The correctly choreographed expression of the genes involved in mediating an immune response is vital for the appropriate immune resolution. The diverse range of immune genes activated at different times over the course of an immune response are largely regulated by common transcription factors such as NF-κB (Saccani, Pantano & Natoli 2001). However despite common activation signals the timing of increased gene expression can vary considerably. As such, genes may be classified as early or primary

responders which show increased activation rapidly following immune activation while others which display delayed activation kinetics are referred to as late or secondary response genes and may be activated hours after the initiating activation signal (Natoli 2009; Ramirez-Carrozzi et al. 2006).

One method which has been used to distinguish primary from secondary response genes is their reliance on new protein synthesis for gene transcription (Ramirez-Carrozzi et al. 2009; Ramirez-Carrozzi et al. 2006; Yamamoto & Alberts 1976). As demonstrated in Chapter 3, the *GM-CSF* gene switches from being a secondary response gene in T cells to being a primary response gene in macrophages. To determine if other immune genes make this switch the translation inhibitor, cycloheximide (CHX) was used to categorise gene expression responses in both T cells and macrophages. Whilst some of the genes examined in this study, such as *Tnfsf9* were clearly independent of new protein synthesis in both cell types others show evidence of switching between the two categories in different cell types.

Like *GM-CSF, IL-23A* switches from being a CHX dependent gene in T cells to being CHX independent in macrophage cells. This indicates that in T cells, regulatory proteins require synthesis before *IL-23A* gene expression can be mediated. These proteins are either not required or are already available without new protein synthesis in macrophages. The *IL-6* and *IL-10* cytokine genes both appear to undergo a switch in activation requirements in the opposite direction with gene expression being independent of new protein synthesis in T cells but dependent in macrophage cells. Several genes including, *IL-1a* and *Tnfsf9* showed enhanced mRNA levels in response to LPS stimulation in the presence of CHX. These transcription responses do not require protein synthesis to occur and in addition mRNA levels of these genes are sustained at a higher level in the CHX samples. Superinduction of mRNA was discussed in Chapter 3 as it was observed in macrophage cells for *GM-CSF* mRNA. The results in this chapter suggest that mRNA superinduction is a common response to CHX treatment. Superinduction of *IL-6* mRNA has previously been reported in lung epithelial cells (Roger, Out, Jansen, et al. 1998). However, neither T cells nor

macrophage cells treated with CHX produced a superinduction response for *IL-6* mRNA suggesting that changes in the gene transcription responses to CHX in different cell types is a recurrent phenomenon. This divergence in gene response may be reflective of differing regulatory requirements, such as transcription factors and chromatin modifying proteins, in different cell types.

From these data, the *IL-23A*, *IL-6* and *IL-10* genes that showed evidence of switching in protein synthesis requirements for activation in the different cell types were selected for further analysis so as to increase our understanding of the mechanisms underlying these switches. These immune regulatory genes have quite diverse functions. The *IL-23A* gene produces the IL-23A (p19) subunit which is related to the p35 subunit of IL-12 and has no biological activity alone (Oppmann et al. 2000). IL-23A binds with the other IL-12 subunit, IL-12B (p40) to form the IL-23 pro-inflammatory cytokine. The distinctly separate role of IL-23 from IL-12 is only beginning to be understood although expression has been associated with late stage and chronic inflammation as well as inflammatory conditions such as arthritis, autoimmune encephalitis and systemic lupus erythematous (Cao et al. 2006; Mus et al. 2010; Smith et al. 2012). IL-23 has been found to induce proliferation of CD4+ T cells (Oppmann et al. 2000) and drives the expansion of a pathogenic population of T cells through the up regulation of inflammatory cytokines including IL-17 and IL-6 contributing to autoimmune pathogenesis (Langrish et al. 2005). IL-6 is a potent pro-inflammatory cytokine which has been found to enhance IL-23 activity and co-operatively up-regulate the production of Th17 cells (Zhou et al. 2007). In addition, IL-6 is produced by a range of cell types and is frequently a therapeutic target in treating inflammatory conditions (Heinrich et al. 2003; Roger, Out, Jansen, et al. 1998; Wongchana & Palaga 2012). Inappropriate *IL-6* gene expression has been associated with a plethora of inflammatory conditions including arthritis and inflammatory bowel disease as well as several cancers (Fishman et al. 1998; Heinrich et al. 2003; Reinecker et al. 1993). In contrast, IL-10 forms part of an auto-inhibitory loop that tempers the transcription and translation of inflammatory cytokines such as IL-6 and IL-23A whilst potentiating TNF-α transcription (Donnelly,

Freeman & Hayes 1995; Fiorentino, Bond & Mosmann 1989; Liu et al. 2009; Moore et al. 2001).

4.3.2 Activation kinetics

It has previously been proposed that the requirement for new protein synthesis before increased gene transcription can be initiated is reflected in delayed activation kinetics, whereby primary response genes which can be actively transcribed without the synthesis of additional proteins respond more rapidly (Ramirez-Carrozzi et al. 2006). Activation kinetics of *IL-23A* gene transcription diverges between the two cell types which may be reflective of differing requirements for new protein synthesis. However, *IL-6* and *IL-10* demonstrated similar biphasic patterns of gene expression in both cell types suggesting that activation kinetics do not always correlate with the requirement for protein synthesis and that protein synthesis requirements may therefore not be the primary driver of activation kinetics.

IL-23A kinetics of activation in response to stimuli occur in two distinctly different patterns in T cells compared to macrophages. In T cells there is a lag time between cell stimulation and the increase in *IL-23A* transcript levels with an exponential increase observed from 2 hours post stimulation continuing out to 24 hours. In macrophage cells, an increase in *IL-23A* mRNA levels was observed as rapidly as 30 minutes following LPS stimulation. This difference in activation kinetics between the two cell types may be partially explained by the divergence in requirement for new protein synthesis. In macrophages, no new protein synthesis is required and an increase in transcription can occur directly following stimulation. In contrast in T cells, following stimulation new proteins, such as transcription factors or chromatin modifiers, must first be synthesised before gene transcription can increase. In contrast, *IL-6* expression is independent of new protein synthesis in T cells, but switches to being dependent on the synthesis of new proteins in macrophages. Despite divergence in the gene expression response in the presence of CHX the activation kinetics of *IL-6* show a

similar trend in T cells and macrophages. A slight initial increase in *IL-6* mRNA is followed by a more prolonged cumulative increase out to 24 hours post stimulation. This biphasic pattern of gene expression is not notably different in T cells compared to macrophages despite the switch in protein synthesis reliance.

In both cell types a biphasic pattern of *IL-10* mRNA levels is evident, this is highly reflective of the important role of *IL-10* as a potent inhibitor of inflammation functioning to prevent the overproduction of inflammatory cytokines and responding to the cytokine environment (Fiorentino, Bond & Mosmann 1989; Moore et al. 2001). An increase in *IL-10* mRNA is detected as soon as 30 minutes following stimulation in both cell types and this fluctuates throughout the time course used in this chapter. It has previously been documented that LPS only induces a modest *IL-10* transcription response and that stimulation in combination with immune complexes produces a more robust response, this was found to be due to enhanced signalling through the MAPK pathway (Lucas et al. 2005). It is likely that the amplitude and activation kinetics of *IL-10* vary in response to different stimuli in order to co-ordinate the most efficient immune response.

The results presented in this chapter clearly demonstrate that there is switching in activation requirements of the same gene between the two cell types examined, with switching between a primary to a secondary response gene in terms of the requirement for new protein synthesis. However this divergence is not necessarily reflected in the activation kinetics of the transcriptional responses to stimulation in the different cell types.

4.3.3 Chromatin structure and remodelling

Alternatively it has been hypothesised that the kinetics of mRNA induction better reflect the requirement for chromatin remodelling for gene activation. As such, genes that are rapidly transcribed are proposed to be held in a more accessible basal chromatin

state while genes that exhibit delayed activation kinetics are held in poised chromatin, requiring the recruitment and activation of chromatin remodelling complexes prior to increased transcription (Natoli 2009; Saccani, Pantano & Natoli 2001). Results presented in this chapter suggest that the basal chromatin accessibility level and the requirement for chromatin remodelling largely correlate with the activation kinetics of gene transcription.

Chromatin accessibility analysis gives an indication of the relative accessibility of the promoter regions to MNase1 digestion which is reflective of accessibility to transcription complex assembly. The ratio used of 'uncut/cut' should give a value of one if the promoter is completely inaccessible, although usually a variable amount of basal accessibility is observed (Cruickshank et al. 2008). The extent of digestion is dependent on the activity and concentration of the nuclease used as well as the conditions of DNA digestion. Therefore while ratio values are not directly comparable between different studies, they should be comparable across different genes in the one study. In Chapter 3, increased chromatin accessibility as determined using the chromatin accessibility assay was shown to be reflective of chromatin remodelling and loss of core histone proteins from the promoter region. The primer sets used to examine promoter accessibility were selected to encompass the DNA sequence occupied by the +1 nucleosome from published literature (refer to Table 2.4, Materials and Methods). Nucleosome phasing at the 5' region of genes has been previously shown to be highly conserved with loss of the +1 nucleosome associated with active gene transcription (Schones et al. 2008).

CHART analysis performed in the EL4 T cells and the RAW 264.7 macrophage cells indicates that prior to stimulation, the *IL-23A* promoter region is relatively inaccessible to MNase1 digestion in T cells and is slightly more accessible in macrophages. Following 4 hours of stimulation relative accessibility was increased in both cell types, which correlates with increased transcription. From the time course data for *IL-23A* mRNA, it appears that increased *IL-23A* gene transcription occurs as early as 30 minutes post stimulation in macrophage cells which correlates with the slightly more

accessible basal chromatin structure in macrophages compared to T cells. Whilst evidence of chromatin remodelling is observed 4 hours post-stimulation in both cell types, it would be interesting to look at chromatin accessibility at earlier time points in order to ascertain whether this increase in promoter accessibility precedes the increased gene transcription, as has been shown previously for the *GM-CSF* gene (Holloway et al. 2003).

In contrast, the *IL-6* gene is held in a relatively accessible basal chromatin structure in macrophages with a ratio of over 1.5 prior to stimulation which is comparable to the activated ratio for the *IL-23A* gene. Following stimulation an increase in accessibility is observed in both cell types. The small, initial increase in mRNA levels at 1-2 hours post-stimuli may be permissible due to the relatively high level of accessibility of the chromatin structure assembled across the promoter region in non-stimulated macrophage cells. The more prolonged, exponential increase in *IL-6* transcript levels seen out to 24 hours may be made possible by increases in promoter accessibility observed in response to 4 hours of stimulation.

The rapid induction of *IL-10* mRNA in response to LPS stimulation of macrophages is in keeping with what has previously been observed in macrophage cells where mRNA transcripts were detectible within minutes of immune stimulation (Zhang, Edwards & Mosser 2006). This prompt increase in mRNA transcript levels is conceivably permissible due to the relatively high level of basal *IL-10* promoter accessibility (ratio of 1.5) which was observed in macrophage cells. An increase in DNase sensitivity at the promoter has previously been reported following only 15 minutes of LPS stimulation (Brightbill et al. 2000; Lucas et al. 2005). A small increase in *IL-10* promoter accessibility was observed following 4 hours of LPS stimulation in macrophages. Whilst the promoter region is located in fairly accessible chromatin prior to stimulation it is possible that fluctuations in promoter accessibility may be observed over a time course, reflective of the biphasic pattern of *IL-10* activation kinetics and that different levels of chromatin accessibility may have been observed had it been measured throughout the time course. In contrast, basal levels of accessibility at the *IL-*

10 promoter in T cells are low. Furthermore no increase in accessibility is observed in response to 4 hours of PI stimulation. This may be reflective of the low levels of *IL-10* mRNA induced by T cells. Alternatively, it is possible that the primer sets used to examine promoter activity were not as optimally positioned to detect promoter activity in both cell types. In particular the primer set chosen for the *IL-10* promoter amplifies a region distant from both the putative NF-κB binding site and the downstream BRG1 binding site (Figure 4.18). Further work may examine changes in promoter accessibility using several primer sets positioned over the length of the promoter region as well as remodelling over a time course.

The analysis of chromatin structure suggests that the basal chromatin accessibility largely dictates a genes ability to respond rapidly to activation signals. In addition the extent of chromatin remodelling correlates with the magnitude of the transcriptional response. Promoter regions which are largely accessible prior to stimulation exhibit faster transcriptional responses than those where basal accessibility is low. This is consistent with the hypothesis that late or secondary responders to immune activation are reliant on chromatin remodelling events before increased gene transcription can occur. The *IL-23A* gene was identified as binding the SWI/SNF family chromatin remodelling enzyme, BRG1 in T cells but has been reported to be SWI/SNF independent in macrophages (Ramirez-Carrozzi et al. 2009). Contrasting this, both the *IL-6* and *IL-10* genes were identified as BRG1 independent in T cells but are reportedly SWI/SNF dependent in macrophage cells (Ramirez-Carrozzi et al. 2009). Although all the genes were found to have a putative BRG1 binding site the location of the BRG1 binding site relative to the transcriptional start site (as depicted in Figure 4.18) varies between different genes. Like *GM-CSF*, *IL-23A* displayed BRG1 binding overlapping the amplicon just upstream of the transcriptional start site. In contrast, both IL-6 and IL-10 have BRG1 binding sites downstream of the +1 nucleosome and the transcriptional start site. This downstream BRG1 binding therefore may not reflect a functional role in chromatin remodelling at the upstream promoter region, but rather a separate function of BRG1.

4.3.4 Regulation by the NF-κB transcription factor, c-Rel

In chapter 3 the difference in CHX response was found to reflect differences in c-Rel nuclear translocation. This is unlikely to be the case for all immune genes as differences in response type are observed within the one cell type. Gene regulation by NF-κB is clearly complex. Data presented here show that c-Rel may act as both a positive and negative regulator of immune gene expression.

The *IL-23A* promoter region contains at least three putative NF-κB binding sites, two of which have been found to interact with c-Rel previously (Carmody et al. 2007; Mise-Omata et al. 2007). ChIP data from this chapter examining c-Rel and Rel-A binding in T cells demonstrates an increase in association of both transcription factors with the *IL-23A* promoter in response to stimulation. Additionally, the *IL-23A* transcription response was inhibited in T cells and macrophages in the presence of the c-Rel inhibitor pentoxifylline (PTX) and in cells derived from c-Rel$^{-/-}$ mice. This suggests that c-Rel is required for *IL-23A* gene transcription in both cell types. *IL-23A* expression has previously been found to be reduced in macrophage cells derived from c-Rel$^{-/-}$ mice as well as macrophages derived from Rel-A$^{-/-}$ mice (Mise-Omata et al. 2007). Both c-Rel and Rel-A have previously been found to be required for *IL-23A* up regulation in dendritic cells and changes in transcription factor binding were attributed to changes in immune response with age (El Mezayen et al. 2009). In c-Rel$^{-/-}$ dendritic cells the production of *IL-23A* is significantly reduced (Carmody et al. 2007).

Both EL4 T cells and RAW 264.7 macrophage cells transfected with a luciferase construct containing approximately 1kb of the *IL-23A* promoter displayed increased luciferase expression in the presence of Rel-A or c-Rel. Previously, RAW 264.7 cells transiently transfected with a reporter plasmid containing the -1180 to +110 fragment of the *IL-23A* promoter region (which encompasses the region used for reporter assays in this chapter) found a dose dependent response in luciferase activity with the addition of

c-Rel (Carmody et al. 2007). It was also found that the Rel-B protein was the only NF-κB transcription factor that does not contribute to increased *IL-23A* gene expression (Carmody et al. 2007). This is most likely reflective of the similar DNA binding motifs of the NF-κB proteins (Kunsch, Ruben & Rosen 1992). In addition several other transcription factors including AP1, SMAD-3, ATF-2 and IRF-3 are known to interact with the *IL-23A* promoter region (Al-Salleeh & Petro 2008; Smith et al. 2012). Although the data presented in this chapter has demonstrated a clear role for c-Rel in *IL-23A* regulation, it is likely that c-Rel binding is just one factor which determines gene transcription events and transcript levels are in fact determined by c-Rel interaction not only with the promoter but also with associated chromatin and transcription factor proteins. The results presented in this chapter provide further support for a requirement for both c-Rel and Rel-A for *IL-23A* expression in macrophage cells as well as T cells.

Analysis of c-Rel and Rel-A association with the *IL-6* promoter region revealed an increase in association of both transcription factors with the promoter region in response to stimulation, suggesting that *IL-6* is directly regulated by c-Rel and Rel-A binding. *IL-6* production in response to stimulation of c-Rel$^{-/-}$ CD4+ T cells and macrophages was consistently higher than that observed in the same cells isolated from wild type mice. Together these results suggest that although c-Rel associates with the *IL-6* promoter region, it has a negative regulatory role. The NF-κB family of transcription factors are thought to be key regulators of *IL-6* expression with NF-κB inhibition with BAY-II abrogating expression responses in macrophage cells (Wongchana & Palaga 2012). Analysis of the human *IL-6* gene has identified an NF-κB binding site, and when this site is mutated promoter activity in response to stimulation is diminished (Libermann & Baltimore 1990) suggesting this site is indispensable for *IL-6* expression.

In EL4 T cells transfected with the *IL-6* promoter reporter construct, co-transfection with either of the NF-κB transcription factors Rel-A or c-Rel resulted in an increase in promoter driven luciferase activity when compared to the CMV control. This suggests

that these transcription factors positively regulated *IL-6* gene transcription in T cells. These results would suggest that the increased transcriptional response in the absence of c-Rel observed in c-Rel$^{-/-}$ cells is attributable to the c-Rel requirement of a negative regulator of *IL-6*. Knockout of c-Rel thereby reduces the availability of this negative regulator, which has a greater influence on *IL-6* gene transcription than the requirement for c-Rel for *IL-6* gene transcription. Alternatively c-Rel could be a direct negative regulator of *IL-6* expression in T cells, but only in a chromatin context not established during transient transfection (Jeong & Stein 1994). On the other hand c-Rel could mediate gene repression through interaction with other regulatory proteins which are not highly expressed in non-stimulated T cells. When transfected into RAW 264.7 macrophage cells the *IL-6* reporter plasmid demonstrated a significant decrease in luciferase activity compared to CMV in the presence of Rel-A and c-Rel which also appeared to have a negative influence on *IL-6* promoter driven luciferase activity. This supports the notion that both Rel-A and c-Rel have a negative regulatory role on *IL-6* gene expression in macrophage cells mediated through direct interaction of the transcription factors with the promoter region. Whilst inducible *IL-6* expression is greater in both T cells and macrophages derived from c-Rel$^{-/-}$ mice and in the presence of c-Rel inhibition the mechanisms by which c-Rel negatively regulates *IL-6* transcription may vary between the two cell types.

The lack of association of either c-Rel or Rel-A with the *IL-10* promoter region suggests that these NF-κB family members do not contribute to *IL-10* gene expression through direct binding to the promoter region. *IL-10* gene transcription has previously been reported to be independent of NF-κB (Bondeson et al. 1999; Brightbill et al. 2000). Although another study has shown that the NF-κB family member NF-κB1 (p50) is able to bind to the *IL-10* promoter region and activate transcription in macrophages cells (Cao et al. 2006). Since chromatin shearing was optimised to give DNA fragments of approximately 500bp in length, it is probable that the promoter primer set used for *IL-10* promoter analysis amplified a region too distant from the NF-κB binding site to detect changes in c-Rel binding to the promoter region.

The heightened *IL-10* induction that was observed in the c-Rel$^{-/-}$ CD4+ and BMDM cells suggest that c-Rel has a negative regulatory effect on *IL-10* expression, however this may not be exerted though c-Rel binding to the *IL-10* promoter region. As c-Rel is known to mediate gene expression responses of a number of immune genes as well as immune regulatory genes such as transcription factors these may in turn influence the *IL-10* response through a flow on effect rather than through direct binding with the promoter region. It has previously been reported that *IL-10* is regulated by transcriptional regulators that themselves are regulated by NF-κB, such as transcription factor STAT1 (Schreiber et al. 2006; Ziegler-Heitbrock et al. 2003).

In EL4 T cells and RAW 264.7 macrophage cells transfected with the *IL-10* promoter construct an increase in *IL-10* promoter activity was observed upon co-transfection with either Rel-A or c-Rel. This is in conflict with the data obtained from c-Rel$^{-/-}$ mice where *IL-10* gene expression was enhanced in the absence of c-Rel. It is possible that in the absence of the associated chromatin structure assembled at the endogenous gene the overexpression of either Rel-A or c-Rel is able to influence *IL-10* promoter activity though the activation of endogenous transcription factors such as STAT1 which co-operatively drive transcription of the reporter construct. As the NF-κB family of transcription factors are well recognised as regulating a broad range of immune response genes, it is likely that the over expression of these transcription factors will not only interact with the co-transfected reporter plasmids but also interact with regulatory regions of the endogenous genes. Such interactions would potentially alter the expression of a number of genes which may also impact on the transcription rate of the reporter construct. Whilst *IL-6* promoter activity in macrophages was negative in the presence of c-Rel, it was expected that this would also be the case when transfected into EL4 T cells, based on the positive effect c-Rel knockout was observed to have on *IL-6* expression. Likewise *IL-10* promoter activity may have been anticipated to decrease when transfected with the c-Rel plasmid. This data suggests that the divergent regulatory ability of c-Rel is due to interaction with other regulatory factors such as the chromatin structure as well as other transcription factors.

The NF-κB transcription factor, c-Rel is documented as having an important role in mediating mature macrophage and T cell function (Gerondakis et al. 2012; Gerondakis et al. 1996; Kontgen et al. 1995). c-Rel functioning as both a positive and negative regulator of cytokine gene expression in different cell types have been reported previously (Gerondakis et al. 1996; Grigoriadis et al. 1996). It is well established that mice lacking c-Rel are defective in mitogenic activation of B and T lymphocytes and display impaired humoural immunity and the results observed in cells isolated from c-Rel$^{-/-}$ mice suggest that this is due to the contribution c-Rel makes to regulating a broad range of cytokine genes (Gerondakis et al. 1996; Kontgen et al. 1995). Previous analysis has found that T cell production of cytokine genes including *IL-3* and *GM-CSF* was diminished in c-Rel$^{-/-}$ cells whilst other genes, such as *IFN-γ* were unaffected (Gerondakis et al. 1996). The work presented in this chapter indicates that c-Rel is able to act as both a positive and negative regulator of gene expression.

The vast majority of studies looking at NF-κB activation of genes have focused on the Rel-A (p65) family member or the NF-κB family as a whole. It is now understood that the individual family members may serve distinctly different roles, in addition more variation is introduced by NF-κB family members binding both as homodimers and as heterodimers with other family members (Smale 2012). The c-Rel transcription factor binds as a homodimer as well as a heterodimer with the NF-κB1 (p50) subunit and Rel-A subunit (Kunsch, Ruben & Rosen 1992). Whilst c-Rel shares an NF-κB binding motif with the Rel-A family member due to the homology of the DNA interacting region of the proteins it is evident from the differences in phenotype of the c-Rel and Rel-A knockout mice that they have distinct function (Baldwin 1996; Kunsch, Ruben & Rosen 1992; Sanjabi et al. 2005). c-Rel is able to recognise all of the binding sites that Rel-A is documented as interacting with, but Rel-A is unable to bind some c-Rel recognition sites which suggest that despite the shared DNA interaction site there is some specificity between the two factors (Schreiber et al. 2006). It is likely that c-Rel competes with other NF-κB transcription factors, including Rel-A, for binding at the NF-κB recognition sites. Perhaps by preventing c-Rel binding by gene knockout this increases the interaction of other NF-κB family members which have a greater ability to

drive gene expression. A further level of complexity may be added to how c-Rel functions to regulate gene expression by the post-translational modification of the c-Rel protein itself (Druker et al. 1994; Fognani et al. 2000; Martin & Fresno 2000).

4.3.5 Conclusions

The data presented in this chapter clearly highlights that immune cytokine genes may be regulated differently in different immune cells. Switching in the requirement for new protein synthesis for gene activation was observed between the two cell types however this did not adequately explain the activation kinetics of the immune genes. The basal chromatin structure established across the regulatory promoter regions correlates with the activation kinetics of gene transcription, with genes that show a rapid increase in gene transcription such as *IL-6*, held in a more permissive basal chromatin structure. In T cells there was evidence of remodelling for the *IL-23A* and *IL-6* genes, however this was not as significant as the remodelling observed at the *GM-CSF* promoter and no evidence of remodelling was observed at the *IL-10* promoter region. Chromatin remodelling was observed in response to stimulation for all genes in macrophage cells suggesting that even when the basal chromatin structure is permissive for gene activation, prolonged activation is sustained through permissive chromatin remodelling events. Whilst the c-Rel transcription factor has a pivotal role in regulating gene expression responses as demonstrated by the changes in gene expression response in the presence of the c-Rel inhibitor PTX or in cells derived from c-Rel$^{-/-}$ mice, it can do so by acting as either a positive or negative regulator of gene expression.

5 Conclusions and Future Directions

This thesis explores the mechanisms of gene regulation of a set of immune genes which fall under the control of the NF-κB transcription factor, c-Rel in both T cells and macrophages to determine if the regulatory mechanisms governing inducible gene expression differ between the two cell types. These genes were classified as either primary or secondary response genes based on their requirement for de novo protein synthesis for increased gene transcription. Results presented here clearly demonstrate that the requirements for inducible gene expression differ between cell types with switching from a primary response gene in one cell type to a secondary response gene in the other. This switching was not uniform in direction for all genes suggesting that the logic behind this switch is not attributable to a single common divergence between T cells and macrophages. The mechanisms underlying this switch may involve the cooperative interaction between a number of proteins such as transcription factors, chromatin structure and remodelling enzymes. Activation kinetics of immune gene transcription are not adequately explained by the requirement for protein synthesis, rather activation kinetics better reflect the basal chromatin environment established at the gene promoter prior to activation. Furthermore, these data demonstrate that c-Rel has a different regulatory effect on different immune genes, functioning as a positive or a negative regulator of gene expression in different contexts.

Aberrant gene expression is associated with a range of immune disorders including inflammatory conditions such as arthritis, asthma and psoriasis as well as proliferative disorders like leukaemia (Heinrich et al. 2003; Langrish et al. 2005; Wills-Karp et al. 1998; Young & Griffin 1986) and the factors that regulate immune responses are attractive targets for intervening in these diseases. However, targeting gene regulatory factors to correct aberrant gene expression also has the potential to de-regulate other genes. Thus a thorough understanding of the interactions between different regulatory factors and gene expression is required before this therapeutic avenue can be explored.

With the advent of genome-wide techniques such as gene expression arrays, ChIP on Chip and ChIP-seq assays we now have a plethora of data documenting gene expression in response to immune stimuli as well as the associated chromatin features. From this data we have gained increased understanding of the chromatin structures that are associated with inducible gene expression and those that mark regions of repressed DNA, as reviewed by Lim et al. (2013). Such studies provide a strong foundation from which single gene studies can build a more thorough understanding of how these common regulatory features interact to mediate inducible gene expression.

The correct spatial and temporal activation of immune genes is crucial for the targeted resolution of immune responses. In order to achieve this some genes are activated more rapidly than others in response to the same stimuli and these have been termed primary and secondary responders (Ramirez-Carrozzi et al. 2006; Yamamoto & Alberts 1976). A possible cause for this delay in activation of some genes compared to others is the reliance on the synthesis of new proteins such as transcriptional activators prior to transcription events occurring (Fowler, Sen & Roy 2011; Ramirez-Carrozzi et al. 2009; Yamamoto & Alberts 1976). In this thesis gene responses were distinguished on this basis by examining gene expression responses in the presence and absence of cycloheximide (CHX), a protein synthesis inhibitor. These data demonstrated that several immune genes expressed in both T cells and macrophages differ in their requirement for new protein synthesis in the different cell types and switching between a primary to secondary response occurs in both directions.

Activation of the *GM-CSF* gene in response to immune stimulation has been studied extensively and it is known to be differentially regulated in different cell types. This thesis confirms that in T cells *GM-CSF* is a secondary response gene whilst in macrophages it is a delayed primary response gene. However this does not reflect a difference in activation kinetics of *GM-CSF* gene transcription, demonstrating that the requirement for new protein synthesis for gene transcription is not always a good predictor of the kinetics of gene response. Similarly, *IL-23A* switches from being a secondary response gene in T cells to being a primary response gene in macrophage

cells. In contrast to *GM-CSF*, *IL-23A* displays divergent activation kinetics between the two cell types. An increase in *IL-23A* mRNA is detected within 30 minutes of macrophage stimulation whereas in T cells transcript levels are not increased until 4 hours post stimulation. This delay in T cells may be partially due to the synthesis of new proteins required for gene activation. The switch from a primary to a secondary response gene was not always one directional. Both *IL-6* and *IL-10* gene expression was independent of new protein synthesis in T cells and switched to being secondary response genes in macrophages. Both *IL-6* and *IL-10* show more biphasic activation kinetics in both cell types suggesting that a requirement for new protein synthesis in macrophages but not T cells does not significantly alter the transcriptional responses.

Western blot analysis of nuclear localisation of c-Rel in both macrophages and T cells suggests that c-Rel nuclear translocation is reliant on the synthesis of new proteins in T cells but not macrophages. These results suggest that the regulation of c-Rel may partially determine classification as a primary or secondary immune response. c-Rel is an attractive target for therapeutic intervention in inflammatory conditions associated with high levels of inflammatory cytokines mediated by c-Rel (Wada 2008; Wada et al. 2012; Wada et al. 2007). The data presented in this thesis suggest that whilst c-Rel is a positive regulator of some inflammatory genes, including *GM-CSF* and *IL-23A*, it is also a negative regulator of others including the potent pro-inflammatory cytokine *IL-6*. Previously c-Rel has been documented as acting as both a positive and negative regulator in different populations of macrophage cells (Grigoriadis et al. 1996). The requirement for gene transcription for c-Rel can be inferred from gene transcription response in the presence of c-Rel inhibitors or in cells derived from c-Rel$^{-/-}$ mice as well as reporter assays as has been done in this thesis. However, these data do not comprehensively demonstrate a direct role for c-Rel in mediating gene transcription levels. Whilst ChIP data in EL4 T cells suggests that c-Rel interacts with not only the *GM-CSF* promoter but also with the *IL-23A* and *IL-6* promoter regions, further studies are required in order to fully elucidate the mechanisms by which c-Rel is able to function as both a positive and negative regulator of gene expression.

Previous work has shown that c-Rel association with the *GM-CSF* promoter is required to mediate chromatin remodelling events in response to immune stimulation (Brettingham-Moore et al. 2005). Future work should therefore examine chromatin remodelling events at the promoter regions in cells acquired from c-Rel$^{-/-}$ mice to determine if chromatin remodelling is permissible in the absence of c-Rel. The potential for interaction between the c-Rel transcription factor and chromatin remodelling complexes would be a useful avenue to explore as it has now been reported that NF-κB transcription factors are required for remodelling events to occur, possibly by facilitating the interaction of chromatin remodelling complexes with specific DNA sequences (Limpert et al. 2013; Poke et al. 2012). In addition the post translational modification of c-Rel, such as phosphorylation may further modify c-Rel function (Druker et al. 1994; Fognani et al. 2000; Martin & Fresno 2000; Perkins 2006).

The analysis of chromatin structure at the proximal promoters of the selected immune genes suggests that the basal chromatin accessibility across the promoter region largely dictates a genes ability to respond rapidly to activation signals. In addition the extent of chromatin remodelling correlates with the magnitude of the response. These findings support the hypothesis that the basal chromatin environment established during cell differentiation determines the genes response to stimuli. There is evidence to suggest that the chromatin structure established at the *GM-CSF* gene is determined through the process of differentiation. In the thymus the human *GM-CSF* gene is held in a silent state at the stage of TCR selection, to minimise the risk of inappropriate activation (Mirabella et al. 2010). During the process of T cell development the human *GM-CSF* locus is progressively activated in terms of both chromatin structure and gene expression (Mirabella et al. 2010). In murine CD4+ T cells, the *GM-CSF* promoter is held in a permissive chromatin structure marked by high levels of histone acetylation (Brettingham-Moore et al. 2008). Inducible gene expression occurs in association with increased promoter accessibility which is reflective of loss of core histone proteins from the *GM-CSF* promoter region (Brettingham-Moore et al. 2005; Brettingham-Moore et al. 2008). There is evidence that this loss of histones is mediated by the BRG1 chromatin remodelling complex (Brettingham-Moore et al. 2005; Brettingham-Moore

et al. 2008) In closely related B cells the *GM-CSF* gene is held in a 'repressive' chromatin structure so that *GM-CSF* mRNA is not increased in response to stimulation (Brettingham-Moore et al. 2008; Sprod 2011). This supports the hypothesis that chromatin structure established at regulatory regions during the differentiation process determines the ability of the gene to respond to inducible stimuli. Further, the work presented in this thesis suggests that the *GM-CSF* gene may be 'primed' for activation in different ways in different cell types. Whilst in macrophage cells *GM-CSF* gene activation is associated with increased promoter accessibility this was found to be independent of loss of core histone protein H3 from the promoter region. Interestingly, whilst an increase in *GM-CSF* mRNA levels was observed with the small change in chromatin accessibility seen in macrophages, the magnitude of this expression response was not a large as that observed in T cells where greater levels of chromatin remodelling are detected. This suggests that the extent of chromatin remodelling may dictate the degree of inducible gene expression. In macrophage cells the *GM-CSF* promoter region is not marked by high levels of histone acetylation prior to activation as it is in T cells. This suggests that chromatin remodelling occurs via different mechanisms in the two cell types as depicted in Figure 5.1. These mechanisms are yet to be fully elucidated and further investigation may include determining if the promoter region is marked by other features such as incorporation of histone variants in macrophage cells. Additionally, once these divergent regulatory features have been characterised, further work may examine how these marks are deposited and replicated during the process of differentiation from HSC to mature immune cell types.

The incorporation of histone variants such as H2A.Z and H3.3 in exchange for canonical histone proteins is reported to destabilise nucleosomes (Jin et al. 2009; Sutcliffe et al. 2009). It is also possible that remodelling occurs via only partial disassembly of the promoter nucleosome so that core H3 is not depleted but perhaps the outer histone proteins, H2A and H2B are lost. The SWI/SNF chromatin remodelling complex has been shown to disassemble nucleosomes in a step-wise manner in which the outer H2A-H2B histone proteins are removed before a slower process removes the H3 and H4 proteins (Dechassa et al. 2010). It has previously been observed with the use

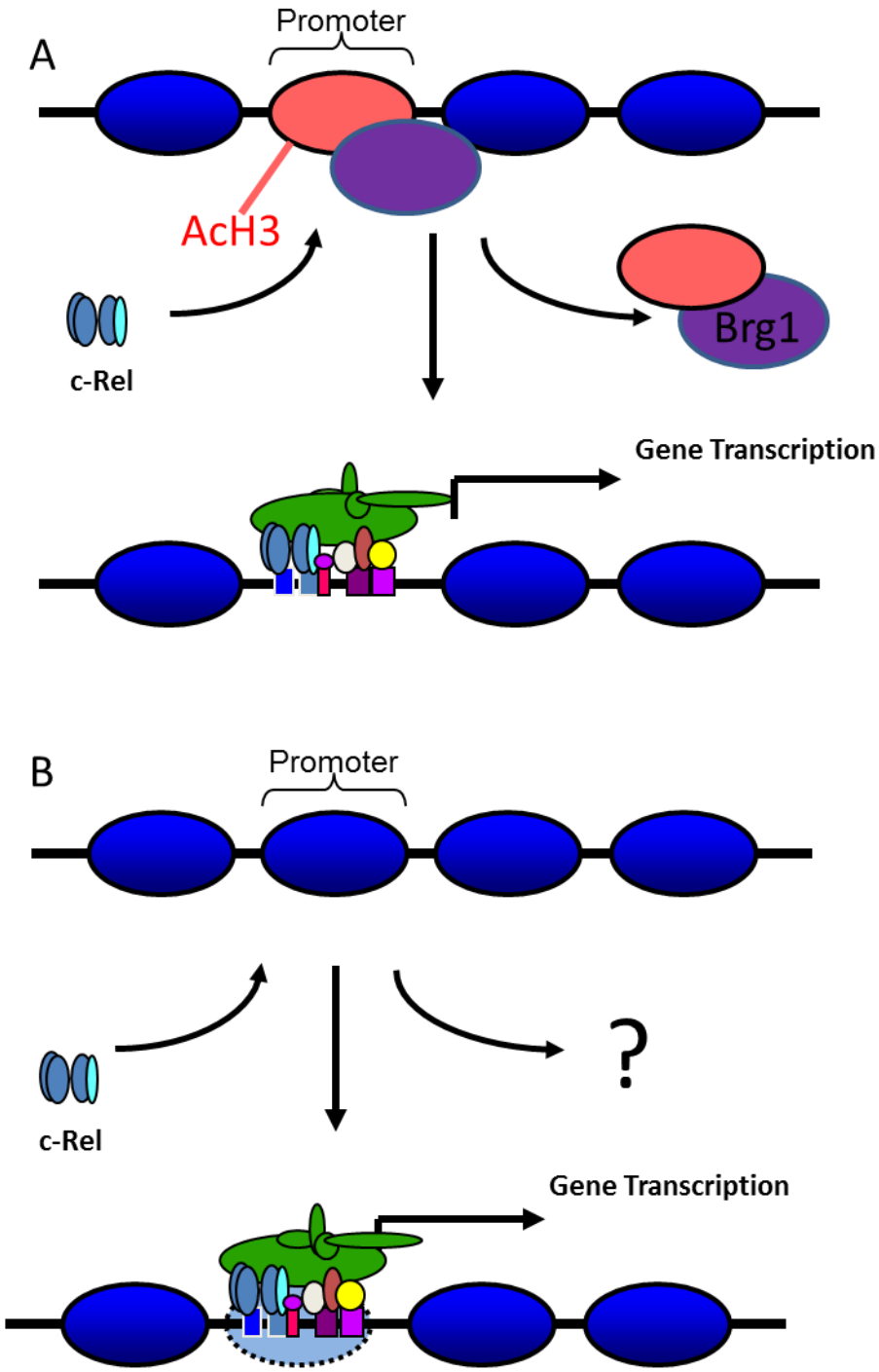

Figure 5.1: Schematic representation of GM-CSF gene activation in T cells compared to macrophages. The GM-CSF promoter in T cells (A) is marked by histone hyperacetylation and the BRG1 protein. Upon activation, histones are depleted from the promote in a c-Rel and BRG1 dependent manner. In macrophages (B) histones at the promoter are not hyperacetylated. Upon activation changes in accessibility occur at the promoter which permit low level gene transcription, but without loss of H3.

of labelled histones, that the H2A and H2B proteins are more frequently exchanged than the H3 and H4 proteins (Bruno et al. 2003) this may enable transcription to occur without complete disruption of the nucleosome, as the H2A-H2B histone dimers are frequently removed during the process of transcription by RNA polymerase II (Kireeva et al. 2002). It would be interesting to determine if gene activation is reliant on the same chromatin remodelling complexes in different cell types. Whilst the *GM-CSF* gene has been found to be activated by BRG1 dependent chromatin remodelling in T cells, *GM-CSF* is reported as a SWI/SNF independent gene in macrophage cells (Ramirez-Carrozzi et al. 2009). Varying the chromatin remodelling complex that mediates gene induction in different cell types would be one way of making gene expression responses cell type specific.

Like *GM-CSF*, *IL-23A* is a pro-inflammatory molecule which was determined through genome-wide analysis to be BRG1 and c-Rel dependent in T cells. Expression analysis in c-Rel$^{-/-}$ cells confirmed a requirement for c-Rel for gene transcription in both cell types. Following 4 hours of stimulation evidence of chromatin remodelling at the *IL-23A* promoter region was seen in both T cells and macrophages correlating with increased gene transcription. However the extent of chromatin remodelling, as well as the magnitude of the transcription response are not as large as that observed for *GM-CSF* in T cells. It would be useful to determine if the *IL-23A* promoter region is marked for activation by high levels of histone acetylation as the *GM-CSF* promoter is in T cells and to determine if the two genes share other chromatin marks such as active or repressive histone methylation. These studies may help to identify common sets of activation requirements for inducible immune gene expression.

In contrast to *GM-CSF* and *IL-23A*, both *IL-6* and *IL-10* promoters are assembled into relatively accessible basal chromatin in macrophage cells which is most likely permissive to early gene transcription events. In T cells basal chromatin was more condensed with relative accessibility ratios closer to 1. While chromatin remodelling was observed in macrophage cells for the *IL-23A*, *IL-6* and *IL-10* gene promoters, basal

chromatin accessibility was often negative (ratio <1) in T cells, with some evidence of chromatin remodelling observed at the *IL-23A* and *IL-6* promoter regions, although not to the same extent as is seen for *GM-CSF*. These data further support the notion that low levels of chromatin remodelling correlate with inducible transcriptional responses of lesser magnitude than those permissible by high levels of chromatin remodelling.

This study could be expanded upon to be more informative if, rather than using just a single primer set to capture remodelling at a small part of the promoter region, a number of primer sets were employed to provide coverage up to 1kb from the transcriptional start site. Negative accessibility data has previously been reported as a mathematical artefact most likely attributable to error in DNA quantitation, and it was found to be neither reproducible nor biologically significant (Liang et al. 2006). However, negative accessibility was generally reported at only a few regions of DNA (amplified by specific primer sets) and when promoter DNA was examined using multiple primer sets, other regions showed evidence of chromatin remodelling (Liang et al. 2006). Additionally, mapping of hypersensitive sites both upstream and downstream of the TSS may be informative in regards to further control of gene transcription through the activation of upstream enhancer and repressor elements. *GM-CSF* gene expression has been shown to be significantly influenced by an upstream enhancer region (Cockerill et al. 1999; Cockerill et al. 1993). Further work may examine how distal regulatory regions are brought into proximity by the chromatin structure to in order to regulate gene expression. This work may further elucidate the significance of the variation in location of the putative BRG1 and NF-κB binding sites relative to the TSS, depicted schematically in Figure 4.18.

Previous work on *IL-10* activation suggests that the activation kinetics and chromatin remodelling may occur within the first 30 minutes of stimulation (Brightbill et al. 2000; Lucas et al. 2005). Whilst a time course was performed in order to examine activation kinetics of *IL-23A*, *IL-6* and *IL-10* mRNA levels, this could be further complemented by a time course to determine the chromatin accessibility associated with changes in mRNA levels. This may be particularly interesting for *IL-6* and *IL-10* where gene

activation has a more biphasic pattern, which may be mediated by waves of transcriptional activators or alternatively fluctuations in chromatin accessibility.

Whilst basal chromatin structure and interaction with transcription factors and chromatin remodelling complexes are crucial for the correct timing of activation of immune genes, the re-setting of this chromatin landscape after the resolution of these gene responses is equally important for immune homeostasis (Poke et al. 2012). Histone reassembly at the *GM-CSF* promoter has previously been found to occur alongside the depletion of c-Rel from the promoter region (Poke et al. 2012). Further work may examine if similar mechanisms of chromatin resetting are utilised in order to resolve the expression of other c-Rel dependent genes.

Genome-wide studies have rapidly expanded our understanding of how different regulatory factors are associated with immune genes both before and after stimulation. These studies must be expanded to encompass interaction between chromatin structure, chromatin modifying complexes and transcription factors in regards to gene expression. Data from this study suggests that association between a transcription factor or chromatin remodelling protein with a gene does not necessarily confer a single function. The schematic depiction of NF-κB binding sites and the chromatin remodelling protein BRG1 relative to the transcriptional start site (Figure 4.18) indicates that for *IL-6* and *IL-10* the potential BRG1 binding site is located downstream of the TSS, this may partially explain the difference in chromatin remodelling and biphasic activation kinetics for these genes. Further work may confirm the interaction between the different regulatory proteins at both proximal and distal regions to further elucidate the regulatory role of these proteins.

This thesis highlights that the activation requirements of the same gene may differ between cell types. Expanding our understanding of the mechanistic switches in activation requirements and the interaction between chromatin structure, transcription factors and chromatin remodelling complexes will make precise targeting of such requirements therapeutically a more viable option.

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APPENDIX A – c-Rel dependent genes

NM_024253	**Nkg7**	**natural killer cell group 7**
NM_009917	**Ccr5**	**chemokine (C-C motif) receptor**
M11024		**hypothetical protein LOC641050**
NM_031395	**Sytl3**	**synaptotagmin-like3**
NM_010370	**Gzma**	**granzyme A**
NM_010288	**Gja1**	**gap junction protein, alpha 1**
BC083101	**C87414**	**expressed sequence C87414**
NM_008607	**Mmp13**	**matrix metallopeptidase 13**
NM_134066	**Akr1c18**	**aldo-keto reductase family 1, member c18**
NM_008147	**Gp49a**	**glycoprotein 49A**
ENSMUST00000020067	**Lilrb4**	**leukocyte immunoglobulin-like receptor, subfamily B, member 4**
NM_001044384	**Timp1**	**tissue inhibitor of metalloproteinase**
NM_008510	**Xcl1**	**chemokine (C motif) ligand 1**
NM_010548	**Il10**	**interleukin 10**
NM_019770	**Tmed2**	**transmembrane emp24 domain trafficking protein 2**
NM_011636	**Plscr1**	**phospholipid scramblase**
NM_001025602	**Il1rl1**	**interleukin 1 receptor-like 1**
NM_010590	**Jub**	**ajuba**
ENSMUST00000101675	**Gm14636**	**predicted gene 14636/100038616**
NM_011454	**Serpinb6b**	**serine (or cysteine) peptidase inhibitor, clade B, member 6b**
NM_028044	**Cnn3**	**calponin 3, acidic**

NM_028849	**Cldnd2**	**claudin domain containing 2**
NM_010554	**Il1a**	**interleukin 1 alpha**
NM_009255	**Serpine2**	**serine (or cysteine) peptidase inhibitor, clade E, member 2**
XM_621704	**Gm5301**	**predicted gene 5301**
NM_153529	**Nrn1**	**neuritin 1**
NM_026075	**Sfrs12ip1**	**SFRS12-interacting protein 1**
NM_001081287	**Mpp7**	**membrane protein, palmitoylated 7**
NM_146218	**Rfwd3**	**ring finger and WD repeat domain 3**
NM_029341	**Capsl**	**calcyphosine-like**
ENSMUST00000114898	**Zc3hav1**	**zinc finger CCCH type, antiviral 1**
ENSMUST00000099550	**Gm10786**	**predicted gene 10786**
NM_001033415	**Shisa3**	**shisa homolog 3**
NM_019656	**Tspan6**	**tetraspanin 6**
NM_025677	**Tsen15**	**tRNA splicing endonuclease 15 homolog (S. cerevisiae)**
NM_010242	**Fut4**	**fucosyltransferase 4**
NM_013754	**Insl6**	**insulin-like**
NM_027091	**Nup35**	**nucleoporin**
NM_029249	**RIKEN**	**RICKEN cDNA**
NM_019549	**Plek**	**pleckstrin**
NM_026122	**Hmgn3**	**high mobility group nucleosomal binding domain 3**
NM_001161515	**Dctd**	**dCMP deaminase**
NM_027154	**Tmbim1**	**transmembrane BAX inhibitor motif containing 1**
ENSMUST00000079237	**Zfp125**	**zinc finger protein 125**
NM_015829	**Slc25a13**	**solute carrier family 25**

		(mitochondrial carrier, adenine nucleotide translocator), member 13
NM_010104	**Edn1**	**endothelin 1**
NM_172301	**Ccnb1**	**cyclin B1**
NM_011369	**Shcbp1**	**Shc SH2-domain binding protein 1**
NM_009004	**Kif20a**	**kinesin family member 20A**
NM_011347	**Selp**	**selectin, platelet**
NM_023645	**Kdelc1**	**KDEL (Lys-Asp-Glu-Leu) containing 1**
NM_019425	**Gnpnat1**	**glucosamine-phosphate N-acetyltransferase 1**
NM_010371	**Gzmc**	**granzyme C**
XR_032134	**Gm2889**	**predicted gene 2889**
ENSMUST00000058104	**Zfp719**	**zinc finger protein 719**
NM_180600	**Ube2q2**	**ubiquitin-conjugating enzyme E2Q (putative) 2**
NM_153116	**Gtpbp10**	**GTP-binding protein 10**
NM_001039251	**Gm7265**	**predicted gene 7265**
XR_031404	**Gm3148**	**predicted gene 3148**
NM_145483	**Zfp160**	**zinc finger protein 160**
NM_026053	**Gemin6**	**gem (nuclear organelle) associated protein 6**
NM_009758	**Bmpr1a**	**bone morphogenetic protein receptor, type 1A**
NM_025664	**Snx9**	**sorting nexin 9**
NM_001004140	**Ckap2**	**cytoskeleton associated protein 2**
NM_016960	**Ccl20**	**chemokine (C-C motif) ligand 20**
NM_145618	**Narg2**	**NMDA receptor-regulated gene 2**

NM_001040699	**Mtmr7**	**myotubularin related protein 7**
NM_011207	**Ptpn3**	**protein tyrosine phosphatase, non-receptor type 3**
NM_145938	**Rpp40**	**ribonuclease P 40 subunit**
NM_001081680	**Zfp72**	**zinc finger protein 72**
NM_001008427	**Gm5595**	**predicted gene**
NM_026958	**RIKEN**	**neural regeneration protein**
NM_030710	**Slamf6**	**SLAM family member 6**
NM_016971	**Il22**	**interleukin 22**
NM_054079	**Iltifb**	**interleukin 10-related T cell-derived inducible factor beta**
BC116235	**Il22**	**interleukin 22**
NM_011990	**Slc7a11**	**solute carrier family 7 (cationic amino acid transporter, y+ system), member 11**
ENSMUST00000099328	**Gm6843**	**predicted gene 6843**
NM_028334	**Nup37**	**nucleoporin 37**
NM_030690	**Rai14**	**retinoic acid induced 14**
NM_013898	**Timm8a1**	**translocase of inner mitochondrial membrane 8 homolog a1 (yeast)**
NM_146231	**Zfp825**	**zinc finger protein 825**
XR_032234	**LOC675534**	**similar to Importin alpha-2 subunit**
NM_144895	**Spg20**	**spastic paraplegia 20, spartin (Troyer syndrome)**
NM_021397	**Zbtb32**	**zinc finger and BTB domain containing**
NM_026507	**Zwilch**	**Zwilch, kinetochore associated, homolog (Drosophila)**
NM_134080	**Flnb**	**filamin, beta**

XR_034872	**Gm7239**	**predicted gene 7239**
NM_011289	**Rpl27**	**ribosomal protein L27**
NM_012012	**Exo1**	**exonuclease 1**
NM_001128096	**Atp13a3**	**ATPase type 13A3**
NM_031252	**Il23a**	**interleukin 23, alpha subunit p19**
NM_080448	**Srgap3**	**SLIT-ROBO Rho GTPase activating protein 3**
NM_009657	**Aldoc**	**aldolase C, fructose-bisphosphate**
NM_026560	**Cdca8**	**cell division cycle associated 8**
NM_009773	**Bub1b**	**budding uninhibited by benzimidazoles 1 homolog, beta (S. cerevisiae)**
NM_027695	**Oxsm**	**3-oxoacyl-ACP synthase, mitochondrial**
NM_001001495	**Tnip3**	**TNFAIP3 interacting protein 3**
NM_176996	**Smo**	**smoothened homolog (Drosophila)**
NM_021782	**Il21**	**interleukin 21**
NM_175406	**Atp6v0d2**	**ATPase, H+ transporting, lysosomal V0 subunit**
NM_013532	**Lilrb4**	**leukocyte immunoglobulin-like receptor, subfamily B, member 4**
NM_009969	**Csf2**	**colony stimulating factor 2 (granulocyte-macrophage)**
NM_019465	**Crtam**	**cytotoxic and regulatory T cell molecule**
NM_007609	**Casp4**	**caspase 4, apoptosis-related cysteine peptidase**

NM_015728	**Slc33a1**	**solute carrier family 33 (acetyl-CoA transporter), member 1**
NM_001142916	**Plod2**	**procollagen lysine, 2-oxoglutarate 5-dioxygenase 2**
NM_013552	**Hmmr**	**hyaluronan mediated motility receptor (RHAMM)**
NM_145825	**Cetn4**	**centrin 4**
NM_015736	**Galnt3**	**UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3**
NM_011636	**Plscr1**	**phospholipid scramblase 1**
NM_001145953	**Lgals3**	**lectin, galactose binding, soluble 3**
NM_026023	**Nudcd2**	**NudC domain containing 2**
NM_175443	**Etnk2**	**ethanolamine kinase 2**
NM_010818	**Cd200**	**CD200 antigen**
NM_013542	**Gzmb**	**granzyme B**
NM_145535	**Sdcbp2**	**syndecan binding protein (syntenin) 2**
NM_053095	**Il24**	**interleukin 24**
NM_011068	**Pex11a**	**peroxisomal biogenesis factor 11 alpha**
NM_001040026	**Sco1**	**SCO cytochrome oxidase deficient homolog 1 (yeast)**
NM_001005421	**Amica1**	**adhesion molecule, interacts with CXADR antigen 1**
NM_134250	**Havcr2**	**hepatitis A virus cellular receptor 2**
NM_172587	**Cdc14b**	**CDC14 cell division cycle 14 homolog B (S. cerevisiae)**

NM_031374	**Tex15**	**testis expressed gene 15**
NM_007759	**Crabp2**	**cellular retinoic acid binding protein II**
NM_033524	**Spred1**	**sprouty protein with EVH-1 domain 1, related sequence**
NM_010287	**Gpr83**	**G protein-coupled receptor**
NM_008737	**Nrp1**	**neuropilin 1**
NM_009012	**Rad50**	**RAD50 homolog (S. cerevisiae)**
NM_023670	**Igf2bp3**	**insulin-like growth factor 2 mRNA binding protein 3**
NM_001033331	**Gas2l3**	**growth arrest-specific 2 like 3**
NM_145706	**Nup43**	**nucleoporin 43**
NM_010128	**Emp1**	**epithelial membrane protein 1**
NM_029797	**Mnd1**	**meiotic nuclear divisions 1 homolog (S. cerevisiae)**
NM_008871	**Serpine1**	**serine (or cysteine) peptidase inhibitor, clade E, member 1**
NM_007962	**Mpzl2**	**myelin protein zero-like 2**
NM_010683	**Lamc1**	**laminin, gamma 1**
NM_011627	**Tpbg**	**trophoblast glycoprotein**
NM_025676	**Mcm8**	**minichromosome maintenance deficient 8 (S. cerevisiae)**
NM_019507	**Tbx21**	**T-box 21**
NM_145381	**Lactb2**	**lactamase, beta 2**
NM_026412	**D2Ertd750e**	**DNA segment**
NM_001045513	**Raph1**	**Ras association (RalGDS/AF-6) and pleckstrin homology domains 1**
NM_010634	**Fabp5**	**fatty acid binding protein 5, epidermal**
NM_013565	**Itga3**	**integrin alpha 3**

NM_008921	**Prim1**	**DNA primase, p49 subunit**
NM_029998	**RIKEN**	**cDNA 6030458C11 gene**
NM_009128	**Scd2**	**stearoyl-Coenzyme A desaturase 2**
NM_027533	**Tspan2**	**tetraspanin 2**
NM_019438	**Ncapg**	**non-SMC condensin I complex, subunit G**
NM_011121	**Plk1**	**polo-like kinase 1 (Drosophila)**
NM_025879	**RIKEN**	**cDNA 2410002O22 gene**
NM_001146081	**Fancb**	**Fanconi anemia, complementation group B**
NM_018782	**Calcrl**	**calcitonin receptor-like**
NM_181542	**Slfn10**	**schlafen 10**
NM_134471	**Kif2c**	**kinesin family member 2C**
NM_011173	**Pros1**	**protein S (alpha)**
NM_212445	**Kdelc2**	**KDEL (Lys-Asp-Glu-Leu) containing 2**
NM_172723	**Adap1**	**ArfGAP with dual PH domains 1**
NM_148933	**Slco4a1**	**solute carrier organic anion transporter family, member 4a1**
NM_027342	**Fam162a**	**family with sequence similarity 162, member A**
NM_008337	**Ifng**	**interferon gamma**
NM_019699	**Fads2**	**fatty acid desaturase 2**
NM_028760	**Cep55**	**centrosomal protein 55**
NM_177884	**AW146020**	**expressed sequence AW146020**
NM_008991	**Abcd3**	**ATP-binding cassette, sub-family D (ALD), member 3**
NM_009445	**Ttk**	**Ttk protein kinase**

NM_027896	**Coasy**	**Coenzyme A synthase**
NM_010790	**Melk**	**maternal embryonic leucine zipper kinase**
NM_026558	**Fam92a**	**family with sequence similarity 92, member A**
NM_027400	**Lman1**	**lectin, mannose-binding,**
NM_026656	**Mcoln2**	**mucolipin 2**
NM_019425	**Gnpnat1**	**glucosamine-phosphate N-acetyltransferase 1**
NM_028986	**Gzf1**	**GDNF-inducible zinc finger protein 1**
NM_008354	**Il12rb2**	**interleukin 12 receptor, beta**
NM_009404	**Tnfsf9**	**tumor necrosis factor (ligand) superfamily, member 9**
NM_028133	**Egln3**	**EGL nine homolog 3 (C. elegans)**
NM_009256	**Serpinb9**	**serine (or cysteine) peptidase inhibitor, clade B, member 9**
NM_018763	**Chst2**	**carbohydrate sulfotransferase 2**
NM_023059	**Sigirr**	**single immunoglobulin and toll-interleukin 1 receptor (TIR) domain**
NM_027838	**Senp8**	**SUMO/sentrin specific peptidase 8**
NM_134133	**RIKEN**	**cDNA 2010002N04 gene**
NM_009977	**Cst7**	**cystatin F (leukocystatin)**
NM_011232	**Rad1**	**RAD1 homolog (S. pombe)**
NM_007457	**Ap1s1**	**adaptor protein complex AP-1, sigma 1**
NM_025518	**Dus2l**	**dihydrouridine synthase 2-like (SMM1, S. cerevisiae)**

NM_011937	**Gnpda1**	**glucosamine-6-phosphate deaminase 1**
NM_011399	**Slc25a17**	**solute carrier family 25 (mitochondrial carrier, peroxisomal membrane protein), member 17**
NM_023284	**Nuf2**	**NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae)**
NM_008495	**Lgals1**	**lectin, galactose binding, soluble 1**
NM_008364	**Il1rap**	**interleukin 1 receptor accessory protein**
NM_029578	**Tgds**	**TDP-glucose 4,6-**
NM_172616	**RIKEN**	**cDNA C330027C09 gene**
NM_007585	**Anxa2**	**annexin A2**
NM_011170	**Prnp**	**prion protein**
NM_175001	**Mrpl22**	**mitochondrial ribosomal protein L22**
NM_021886	**Cenph**	**centromere protein H**
NM_029942	**Prelid2**	**PRELI domain containing 2**
NM_010892	**Nek2**	**NIMA (never in mitosis gene a)-related expressed kinase 2**
NM_198127	**Abi2**	**abl-interactor 2**
NM_025384	**Dnajc15**	**DnaJ (Hsp40) homolog, subfamily C, member 15**
NM_177684	**Zfp637**	**zinc finger protein**
NM_028006	**Tube1**	**epsilon-tubulin 1**
NM_028304	**Pus10**	**pseudouridylate synthase**
BC158017	**Cdc42bpa**	**CDC42 binding protein kinase alpha**

NM_176993	**Mpzl3**	**myelin protein zero-like 3**
NM_148928	**Gtf3c5**	**general transcription factor IIIC, polypeptide 5**
NM_020559	**Alas1**	**aminolevulinic acid synthase 1**
NM_010720	**Lipg**	**lipase, endothelial**
NM_015804	**Atp11a**	**ATPase, class VI, type 11A**
NM_199007	**Sgol2**	**shugoshin-like 2 (S. pombe)**
NM_030013	**Cyp20a1**	**cytochrome P450, family 20, subfamily A, polypeptide 1**
NM_026644	**Agpat4**	**1-acylglycerol-3-phosphate O-acyltransferase 4 (lysophosphatidic acid acyltransferase, delta)**
NM_008445	**Kif3c**	**kinesin family member 3C**
NM_173762	**Cenpe**	**centromere protein E**
NM_145480	**Rfc4**	**replication factor C (activator 1) 4**
NM_016965	**Nckap1**	**NCK-associated protein 1**
NM_009015	**Rad54l**	**RAD54 like (S. cerevisiae**
NM_145924	**Cenpi**	**centromere protein I**
NM_173750	**RIKEN**	**cDNA 2700007P21 gene**
NM_026053	**Gemin6**	**gem (nuclear organelle) associated protein 6**
NM_175193	**Golim4**	**golgi integral membrane protein 4**
NM_016918	**Nudt5**	**nudix (nucleoside diphosphate linked moiety X)-type motif 5**
NM_024261	**RIKEN**	**cDNA 1700052N19 gene**
NM_012048	**Polk**	**polymerase (DNA directed), kappa**

NM_133838	**Ehd4**	**EH-domain containing 4**
NM_176982	**Fbxo48**	**F-box protein 48**
NM_009517	**Zmat3**	**zinc finger matrin type 3**
NM_026221	**Ppfibp1**	**PTPRF interacting protein, binding protein 1 (liprin beta 1)**
NM_198605	**RIKEN**	**cDNA F630043A04 gene**
NM_027346	**Ccdc44**	**coiled-coil domain containing 44**
NM_173757	**Mrps27**	**mitochondrial ribosomal protein S27**
NM_181410	**Gtf2h3**	**general transcription factor IIH, polypeptide 3**
NM_018861	**Slc1a4**	**solute carrier family 1 (glutamate/neutral amino acid transporter), member 4**
NM_007796	**Ctla2a**	**cytotoxic T lymphocyte-associated protein 2 alpha**
NM_025979	**Mastl**	**microtubule associated serine/threonine kinase-like**
NM_028232	**Sgol1**	**shugoshin-like 1 (S. pombe)**
NM_026178	**Mmd**	**monocyte to macrophage differentiation-associated**
NM_001127338	**Aldh7a1**	**aldehyde dehydrogenase family 7, member A1**
NM_183046	**Kif20b**	**kinesin family member 20B**
NM_025935	**Tbc1d7**	**TBC1 domain family, member 7**
NM_001080995	**RIKEN**	**cDNA 4632434I11 gene**
NM_145460	**Oxnad1**	**oxidoreductase NAD-binding domain containing 1**
NM_153780	**RIKEN**	**cDNA 2610044O15 gene**
NM_009811	**Casp6**	**caspase 6**

NM_022324	**Sdf2l1**	**stromal cell-derived factor 2-like 1**
NM_001113179	**Bub1**	**budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)**
NM_001114663	**Plcl1**	**phospholipase C-like 1**
NM_001135149	**Slc39a8**	**solute carrier family 39 (metal ion transporter), member 8**
NM_010260	**Gbp2**	**guanylate binding protein 2 // 3**
NM_008925	**Prkcsh**	**protein kinase C substrate 80K-H**
NM_010049	**Dhfr**	**dihydrofolate reductase**
NM_029720	**Creld2**	**cysteine-rich with EGF-like domains 2**
NM_029017	**Mrpl47**	**mitochondrial ribosomal protein L47**
NM_175002	**Mmgt2**	**membrane magnesium transporter 2**
NM_172653	**Slc39a10**	**solute carrier family 39 (zinc transporter), member 10**
NM_173763	**Ccbl2**	**cysteine conjugate-beta lyase 2**
NM_146097	**Cbwd1**	**COBW domain containing 1**
NM_173745	**Dusp18**	**dual specificity phosphatase 18**
NM_175563	**Prr11**	**proline rich 11**
NM_011849	**Nek4**	**NIMA (never in mitosis gene a)-related expressed kinase 4**

APPENDIX B – Gene expression time course data relative to GAPDH

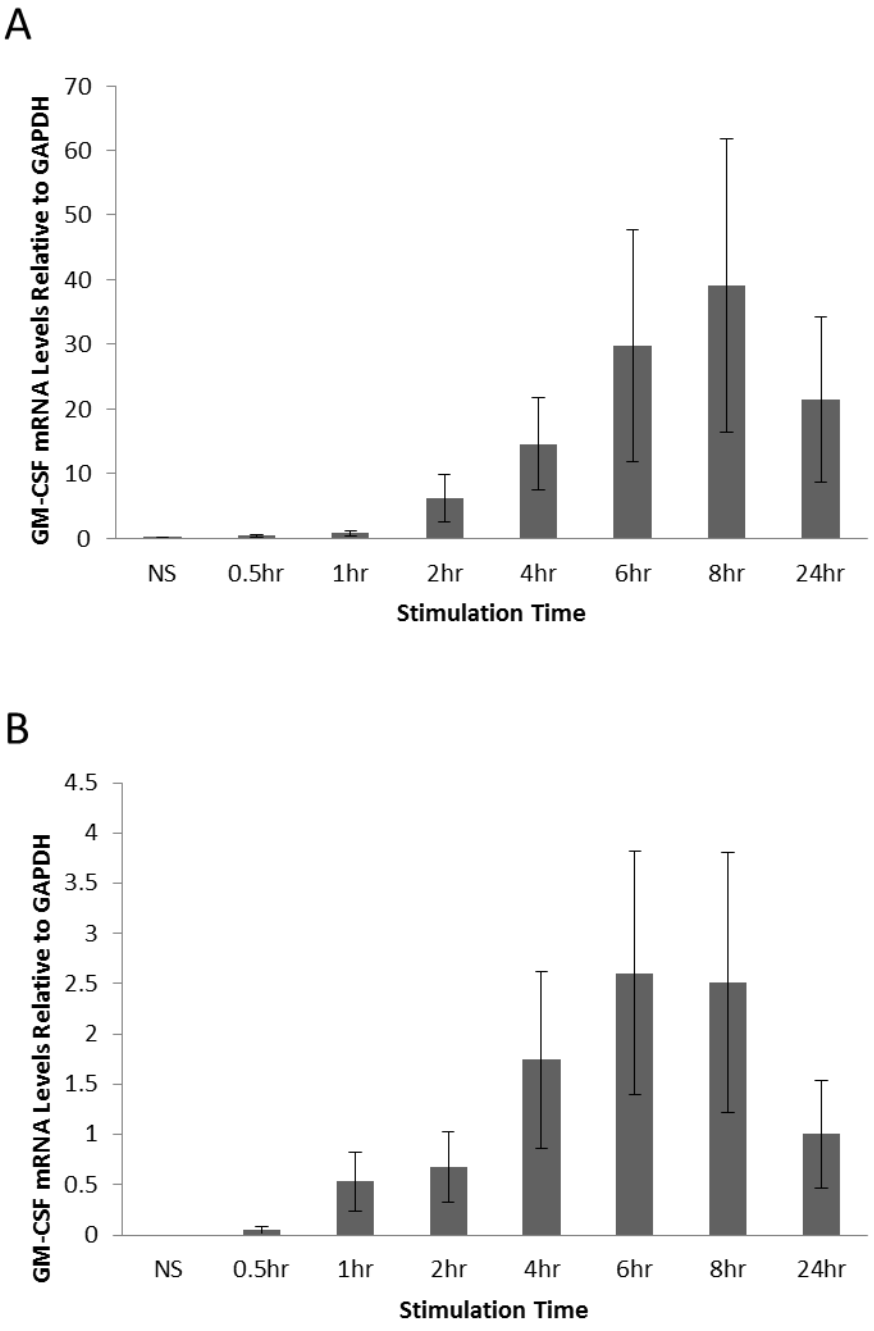

Figure B.1: Activation kinetics of the GM-CSF gene in T cell and macrophage cell lines. (A) Murine EL4 T cells were incubated with PI and (B) RAW 264.7 macrophages were incubated with LPS. Cells were either left unstimulated (NS) or stimulated for the indicated times. In each case, RNA was isolated and GM-CSF mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown.

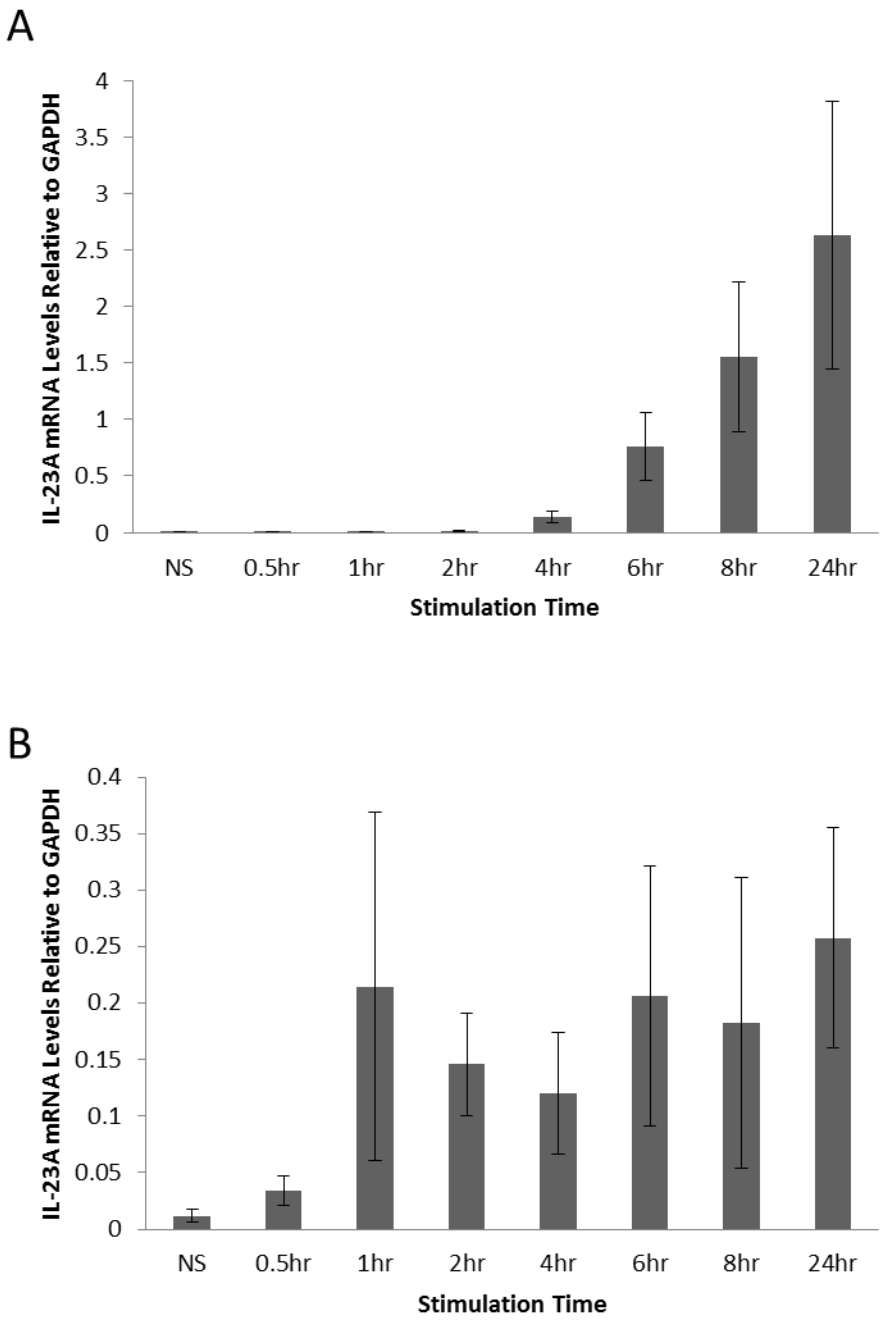

Figure B.2: Activation kinetics of the IL-23A gene in T cell and macrophage cell lines. (A) Murine EL4 T cells were incubated with PI and (B) Murine RAW 264.7 macrophage cells were incubated with LPS. Cells were either left non-stimulated (NS) or stimulated for the times indicated. In each case, RNA was isolated and IL-23A mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown.

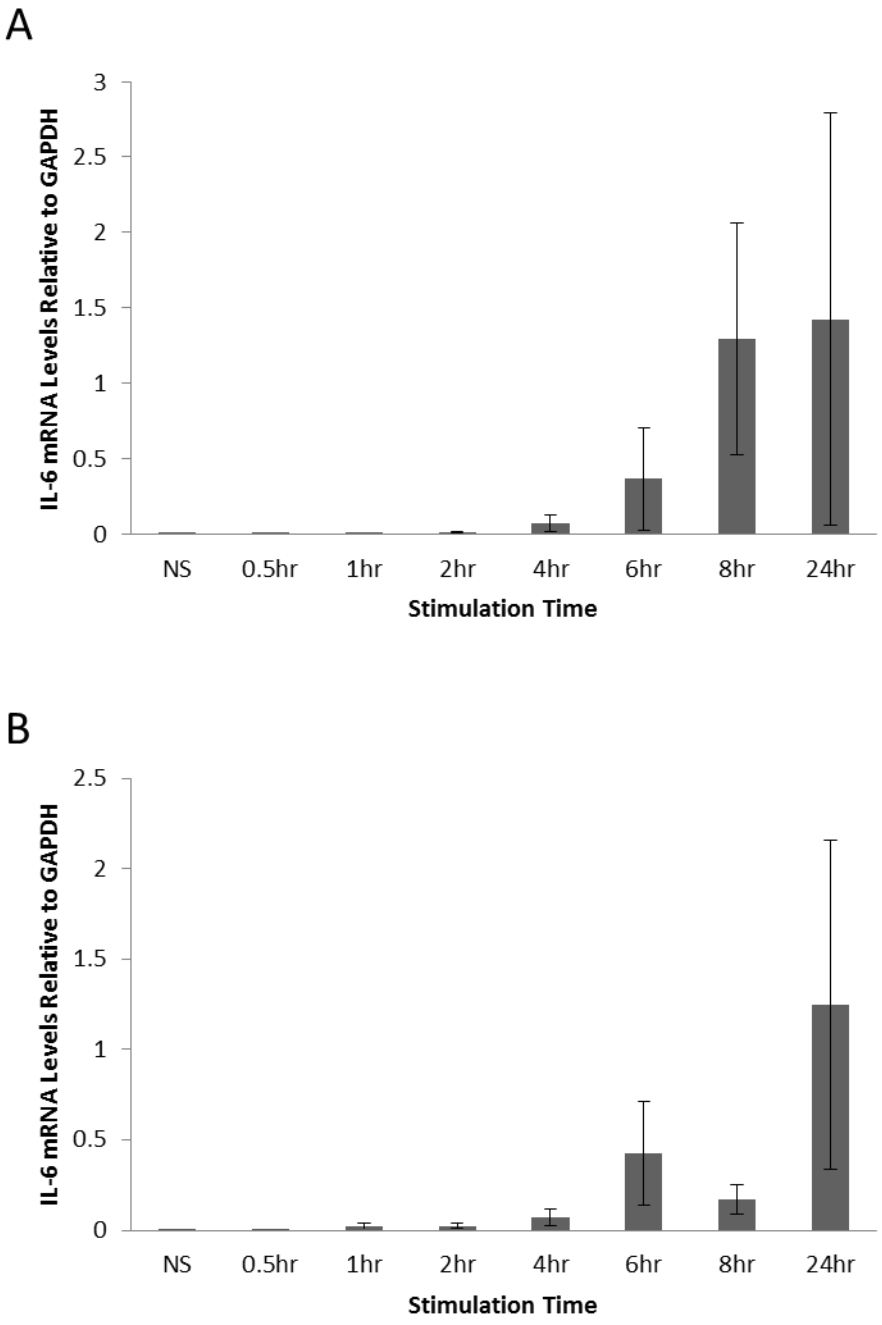

Figure B.3: Activation kinetics of the IL-6 gene in T cell and macrophage cell lines. (A) Murine EL4 T cells were incubated with PI and (B) Murine RAW 264.7 macrophage cells were incubated with LPS. Cells were either left non-stimulated (NS) or stimulated for the indicated times. In each case, RNA was isolated and IL-6 mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown.

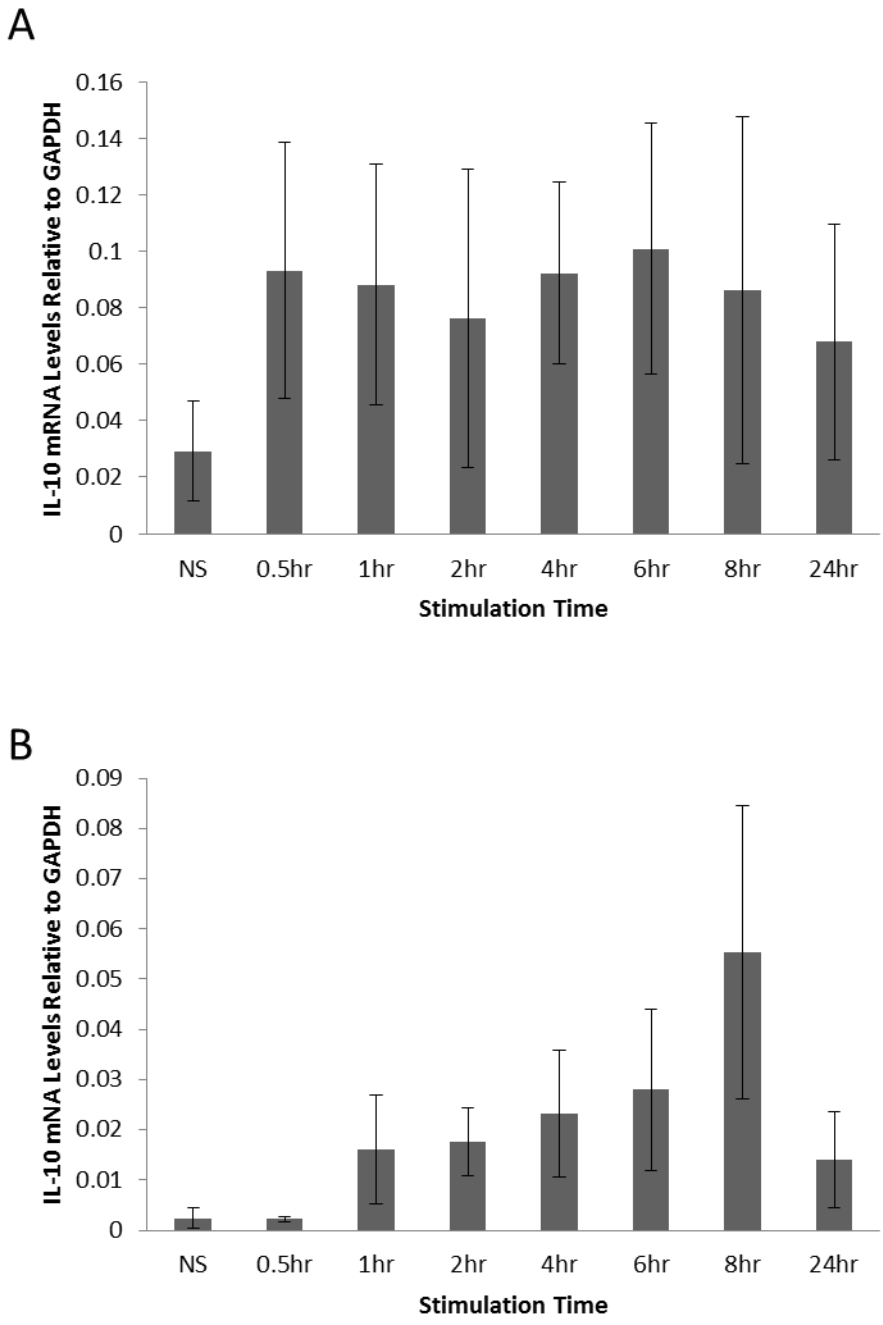

Figure B.4: Activation kinetics of the IL-10 gene in T cells and macrophage cell lines. (A) murine EL4 T cells were incubated with PI and (B) Murine RAW 264.7 macrophage cells were incubated with LPS. Cells were either left non-stimulated (NS) or stimulated for the times indicated. In each case, RNA was isolated and IL-10 mRNA levels determined by RT-qPCR and normalised to GAPDH. Mean and SEM of at least 3 replicates is shown.